university of groningen / faculty of science and engineering / analytical biochemistry

Bioanalysis of Biopharmaceuticals and Biomarkers by Liquid Chromatography – Mass Spectrometry

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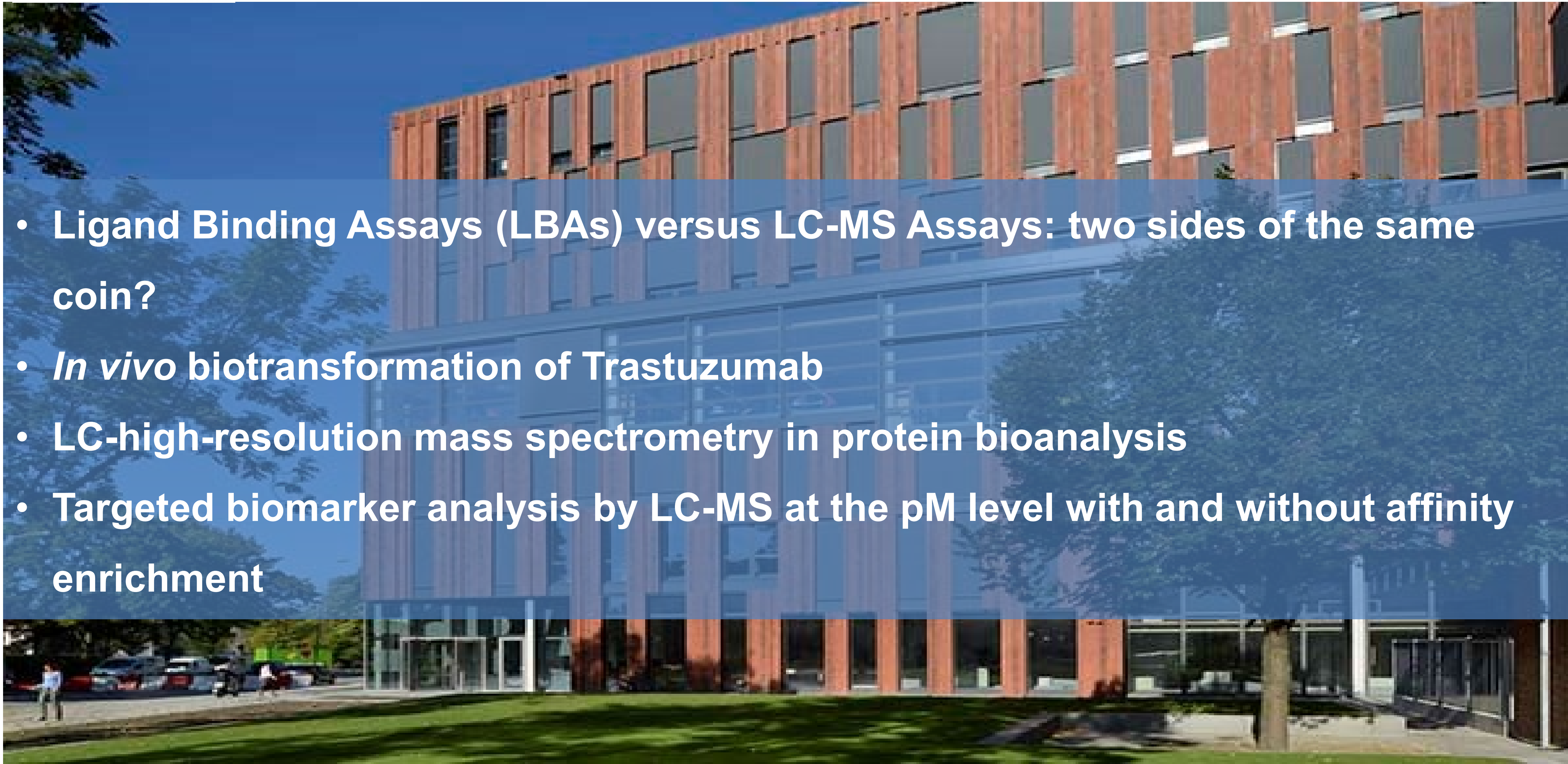
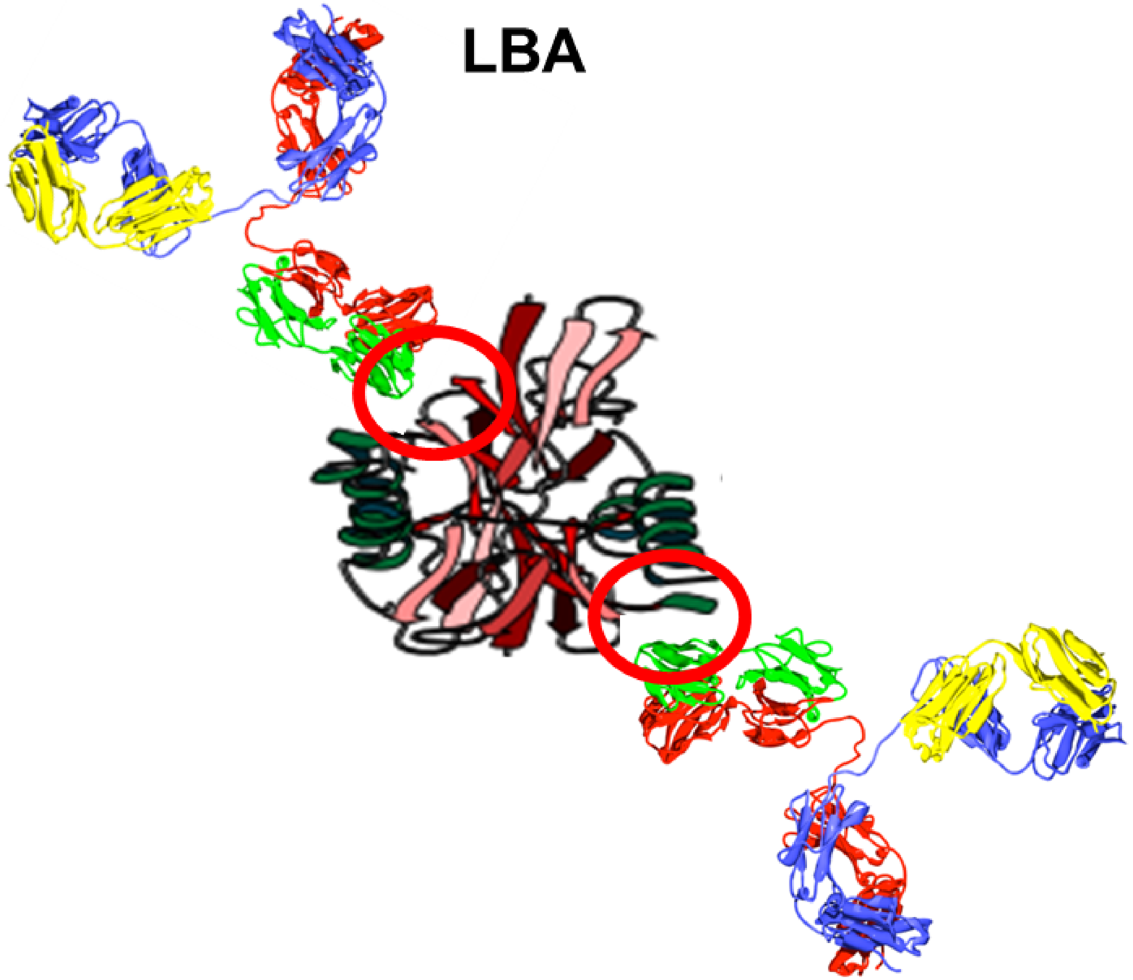
European Research Institute: Biology of Ageing

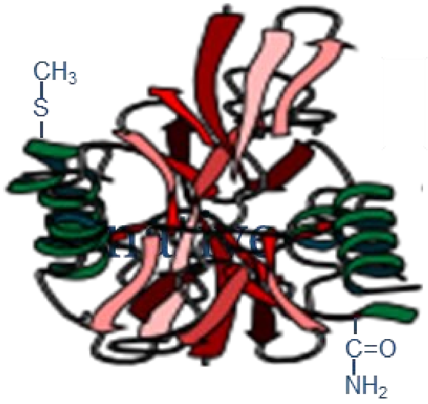


- Ligand Binding Assays (LBAs) versus LC-MS Assays: two sides of the same coin?
- *In vivo* biotransformation of Trastuzumab
- LC-high-resolution mass spectrometry in protein bioanalysis
- Targeted biomarker analysis by LC-MS at the pM level with and without affinity enrichment

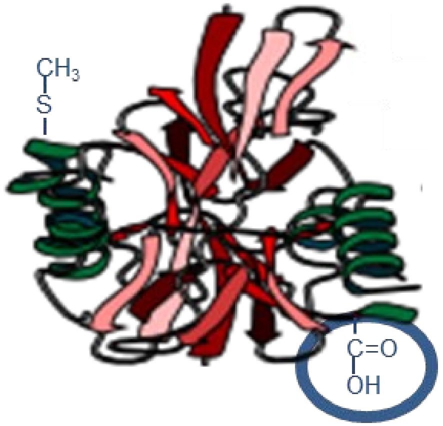
Ligand Binding Assays (LBAs) and LC-MS Assays



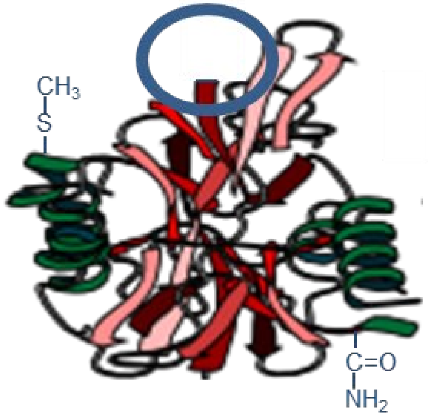
Are they two sides of the same coin?



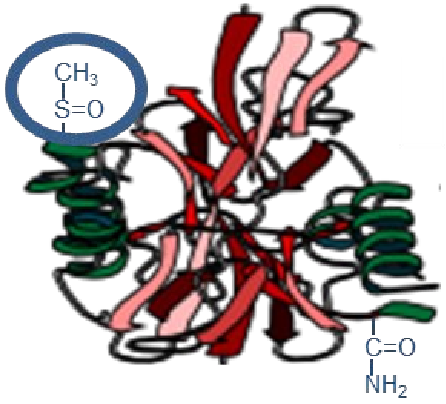
Deamidation



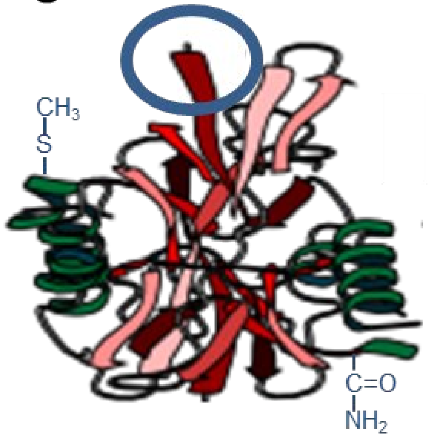
Proteolysis



Oxidation

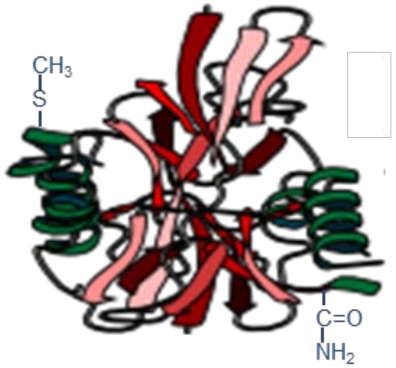


Change in 3D Structure

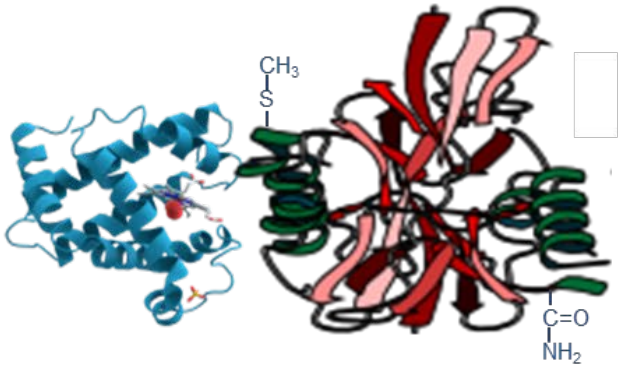


LBAs may be affected by competition for binding

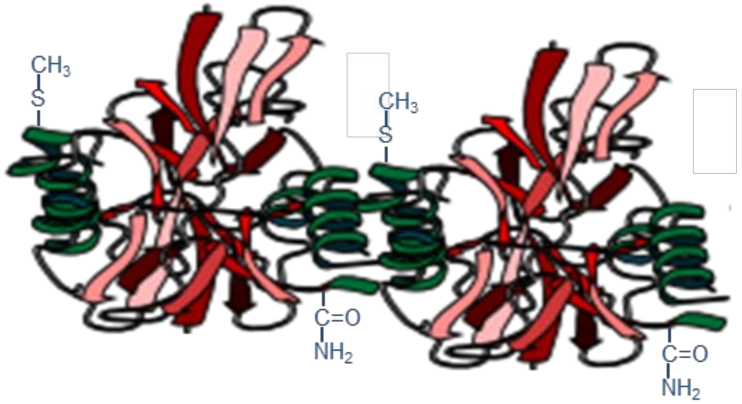
Free Form



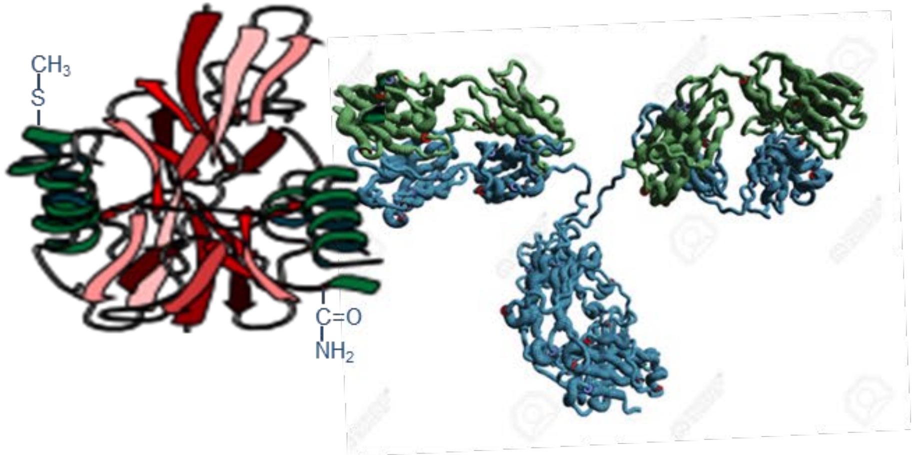
Target-Bound Form



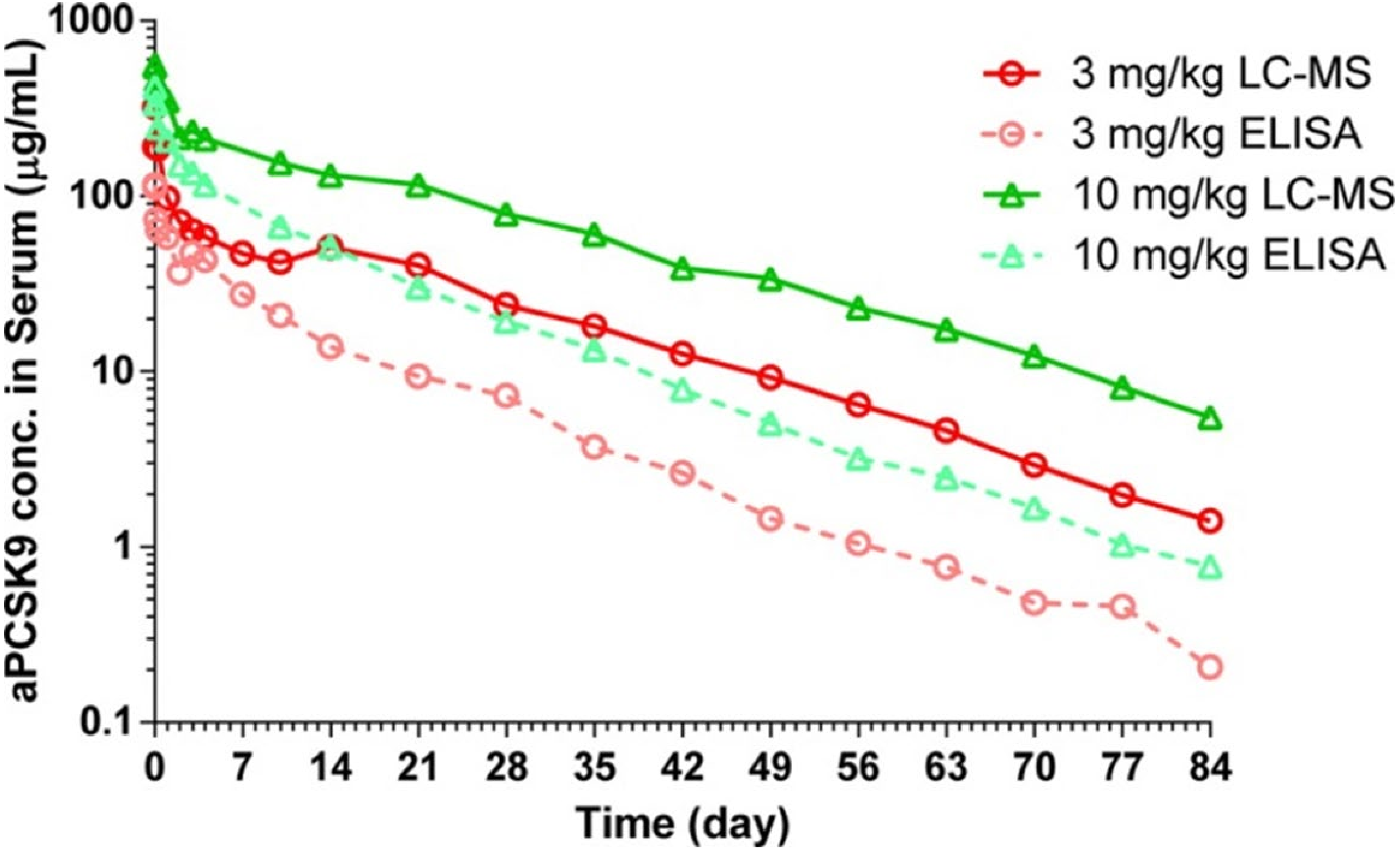
Dimerized Form



ADA-Bound Form

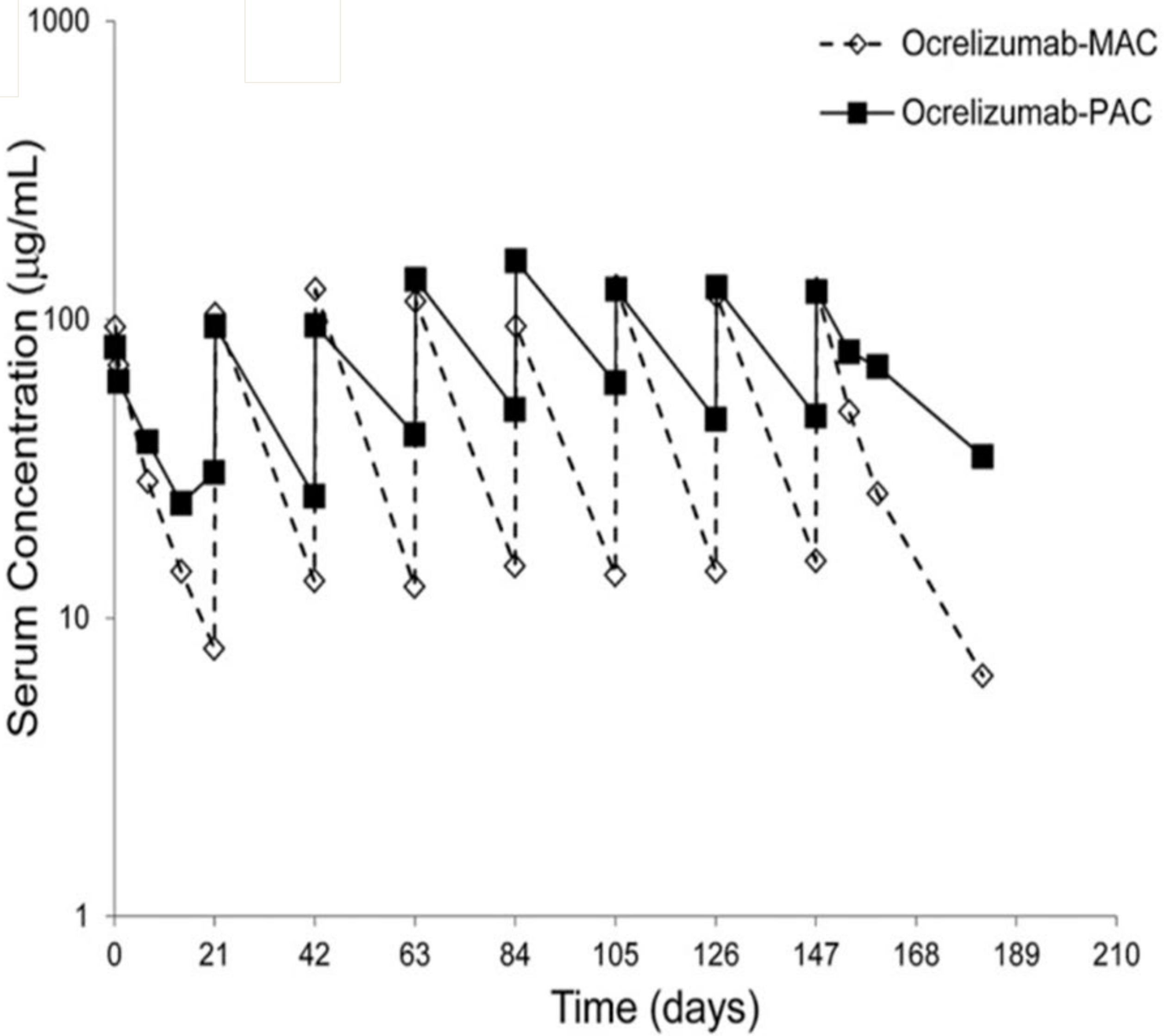


LC-MS versus ELISA



Zhang et al. Anal Chem, 86, 8776-8784 (2014)

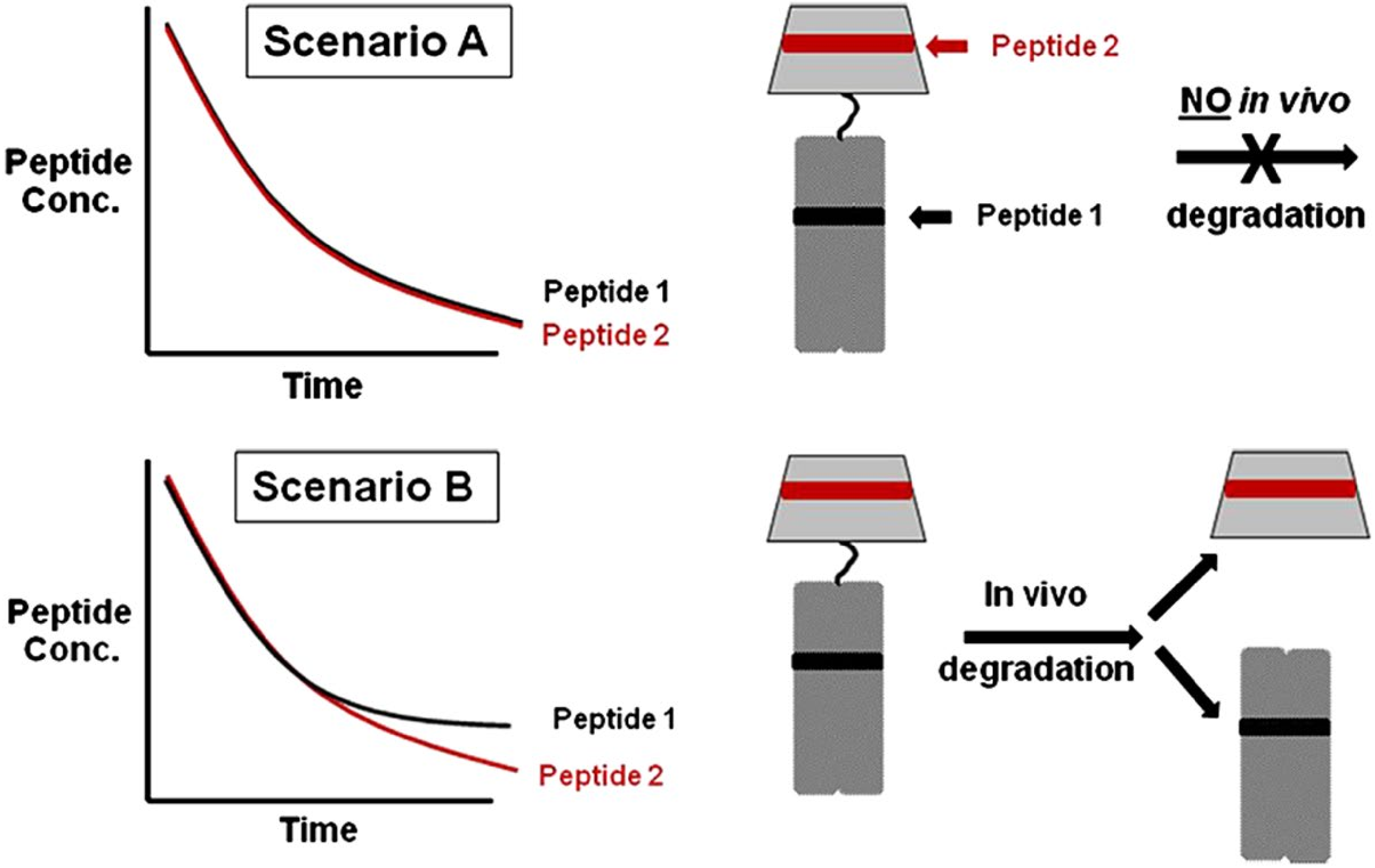
Different Immunocapture Agents



MAC: monoclonal antibody
PAC: polyclonal antibody

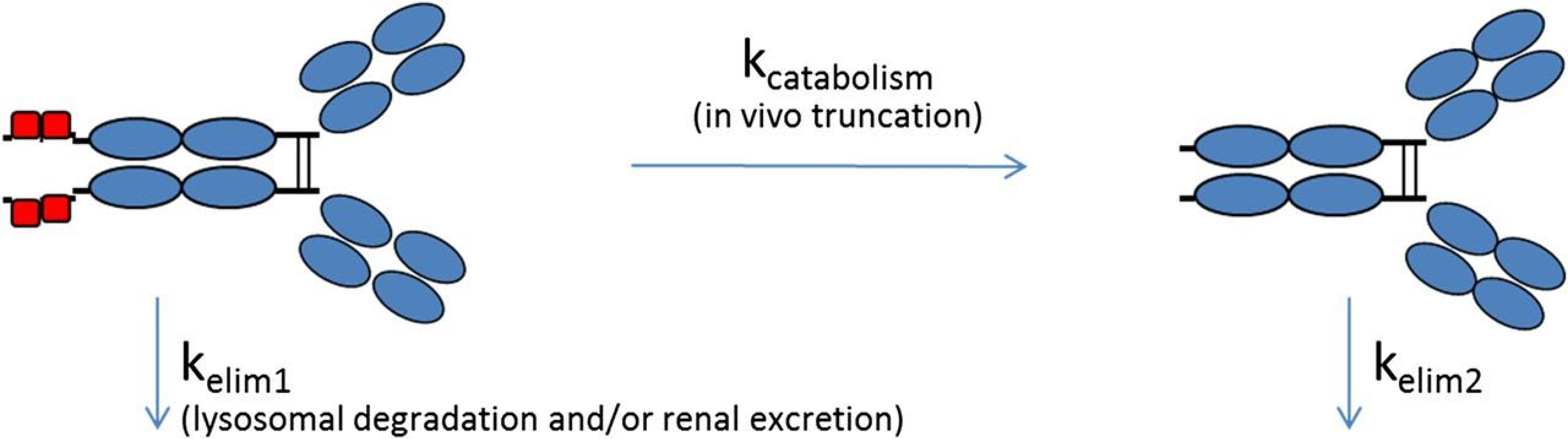
Fischer et al. mAbs, 4, 623-631 (2012)

In vivo Processing/Degradation



Furlong, et al. Biomedical Chromatography, 26, 1024-1032 (2012)

In vivo Biotransformation of Biopharmaceuticals



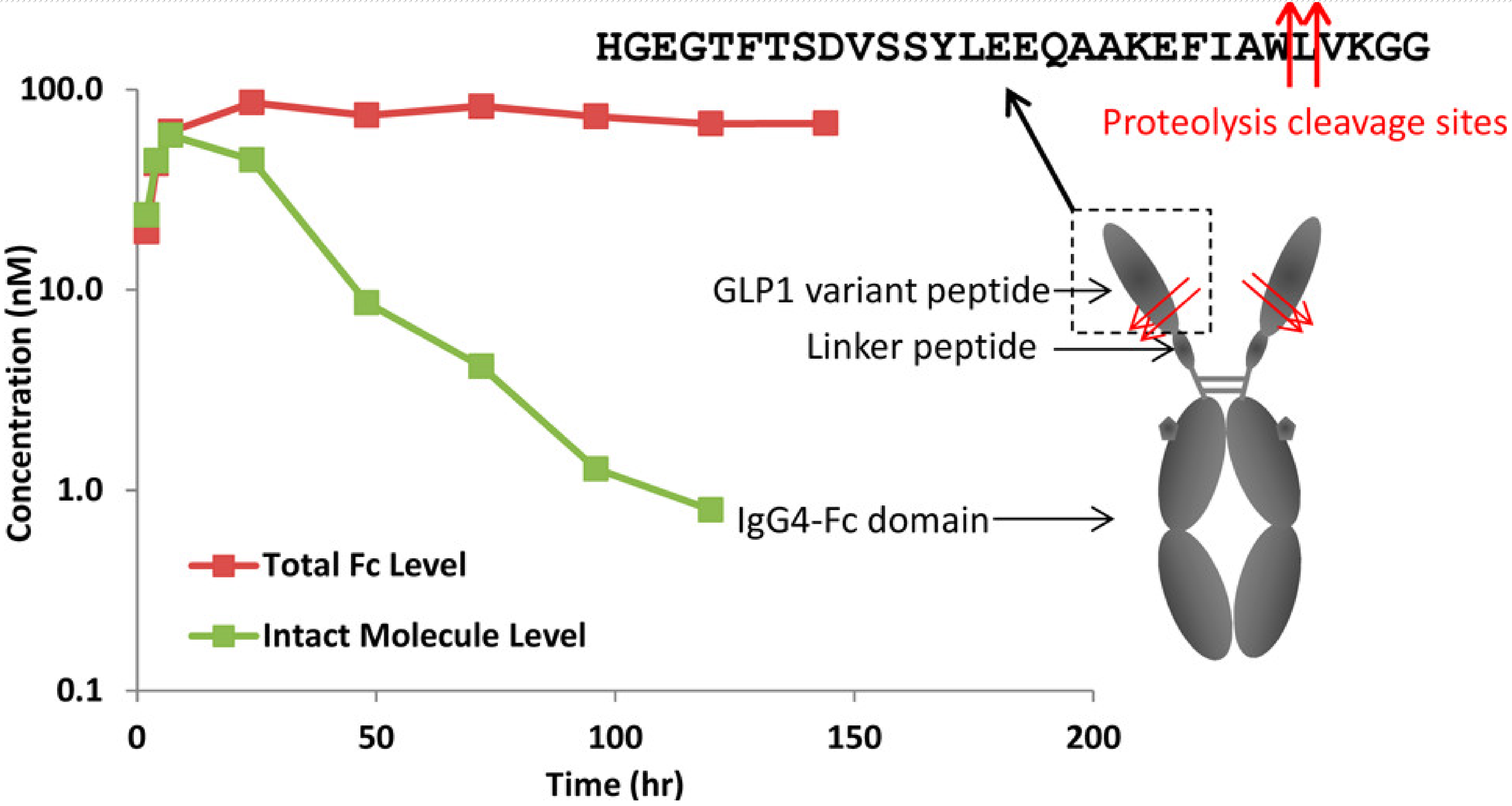
$k_{catabolism}$
(in vivo truncation)

k_{elim1}
(lysosomal degradation and/or renal excretion)

k_{elim2}

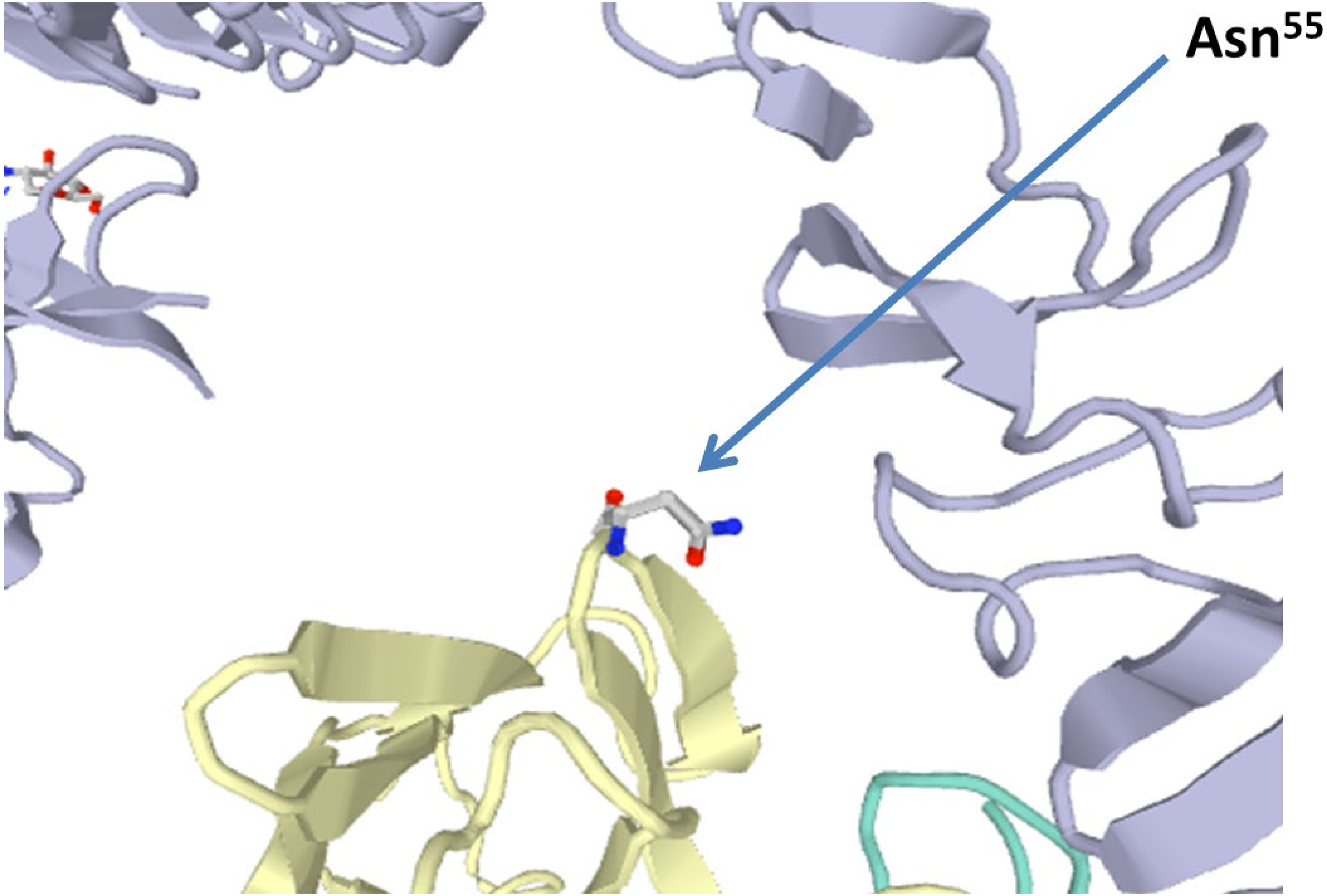
Hall, Drug metabolism and disposition: the biological fate of chemicals **2014**, 42, 1873-1880.

In vivo Biotransformation analyzed by LC-MS

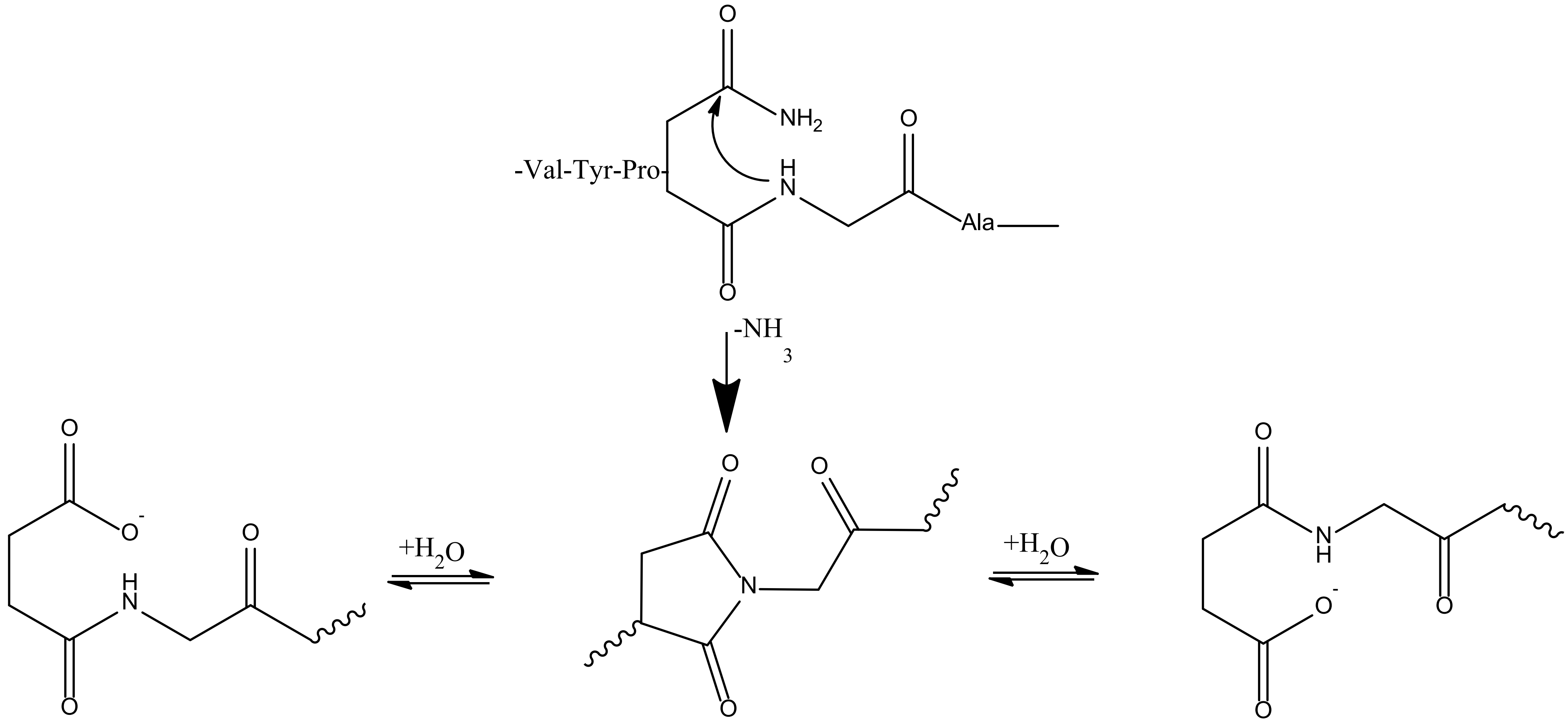


Kang et al., *Anal. Chem.* **2017,** 89, 6065-6075.

The Trastuzumab-Her2 Complex



Deamidation of Trastuzumab



L-Asn-peptide

L-Asp-peptide

L-Imide-peptide

L-isoAsp-peptide

Bischoff & Kolbe, J. Chrom. B, 662, 261-278 (1994)

Selection of Signature Peptides

Asn55

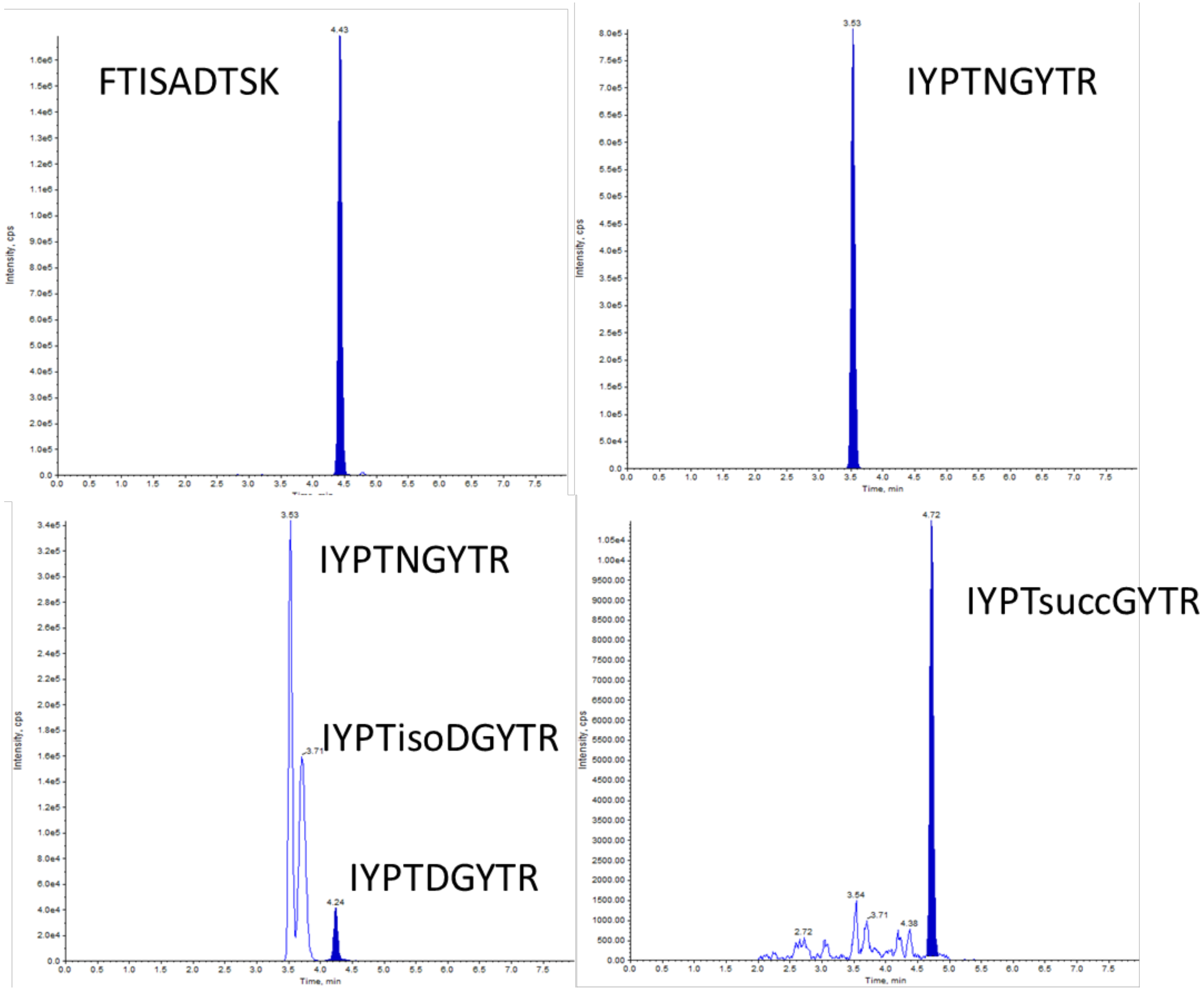
Trastuzumab heavy chain

1 EVQLVESGGG LVQPGGSLRL SCAASGFNIK DTYIHWVRQA PGKGLEWVAR IYPT**N**GYTRY
61 ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCSRWG GDGFYAMDYW GQGTLVTVSS
121 ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS
181 GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP PKSCDKTHTC PPCPAPELLG
241 GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY
301 NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPSRD
361 ELTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTPP VLDSDGSFFL YSKLTVDKSR
421 WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K

Trastuzumab light chain

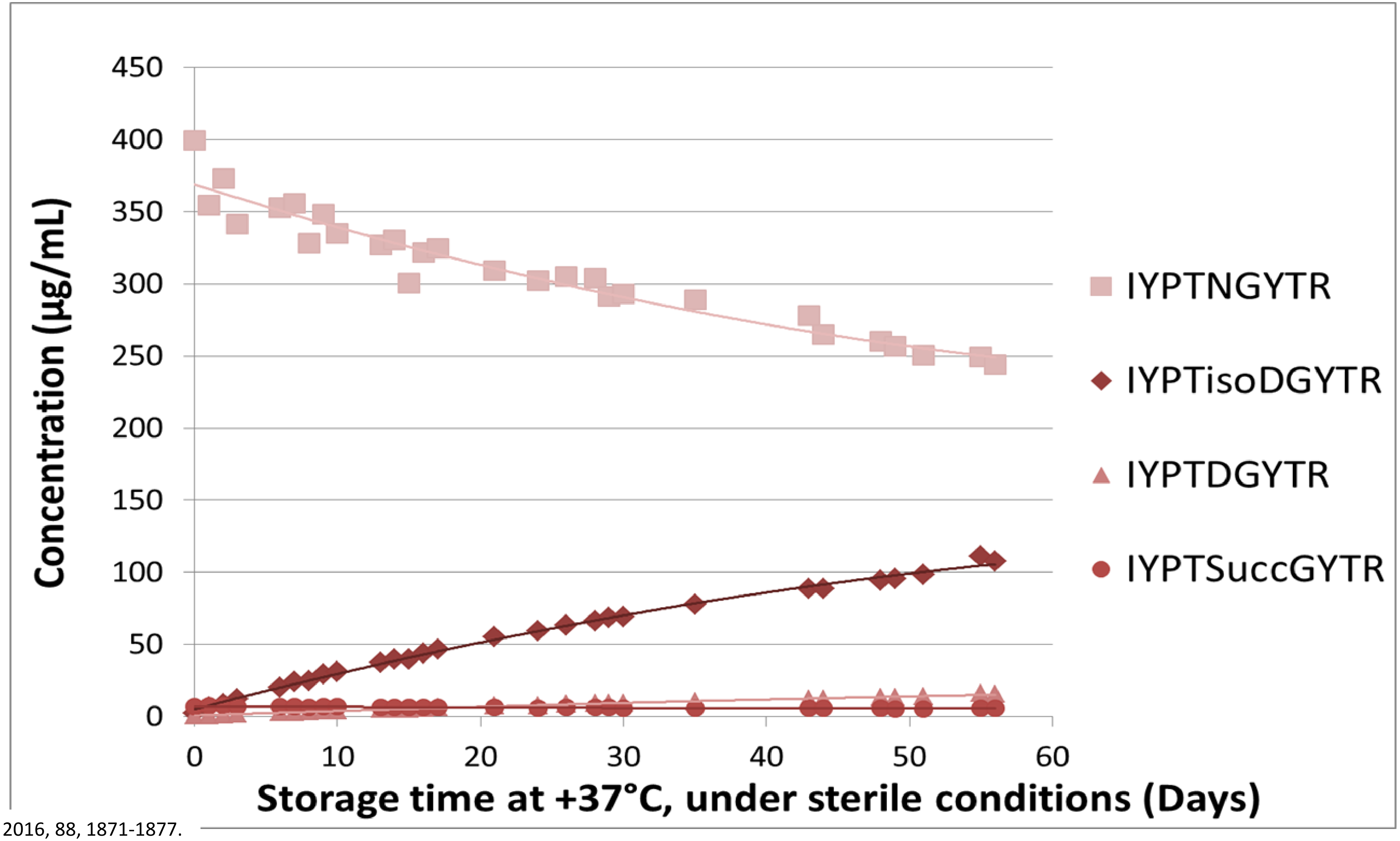
1 DIQMTQSPSS LSASVGDRVT ITCRASQDVN TAVAWYQQKP GKAPKLLIYS ASFLYSGVPS
61 RFSGSRSGTD FTLTISSLQP EDFATYYCQQ HYTTPPTFGQ GTKVEIKRTV AAPSVFIFPP
121 SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT
181 LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC

Probing Deamidation at Asn^{55}



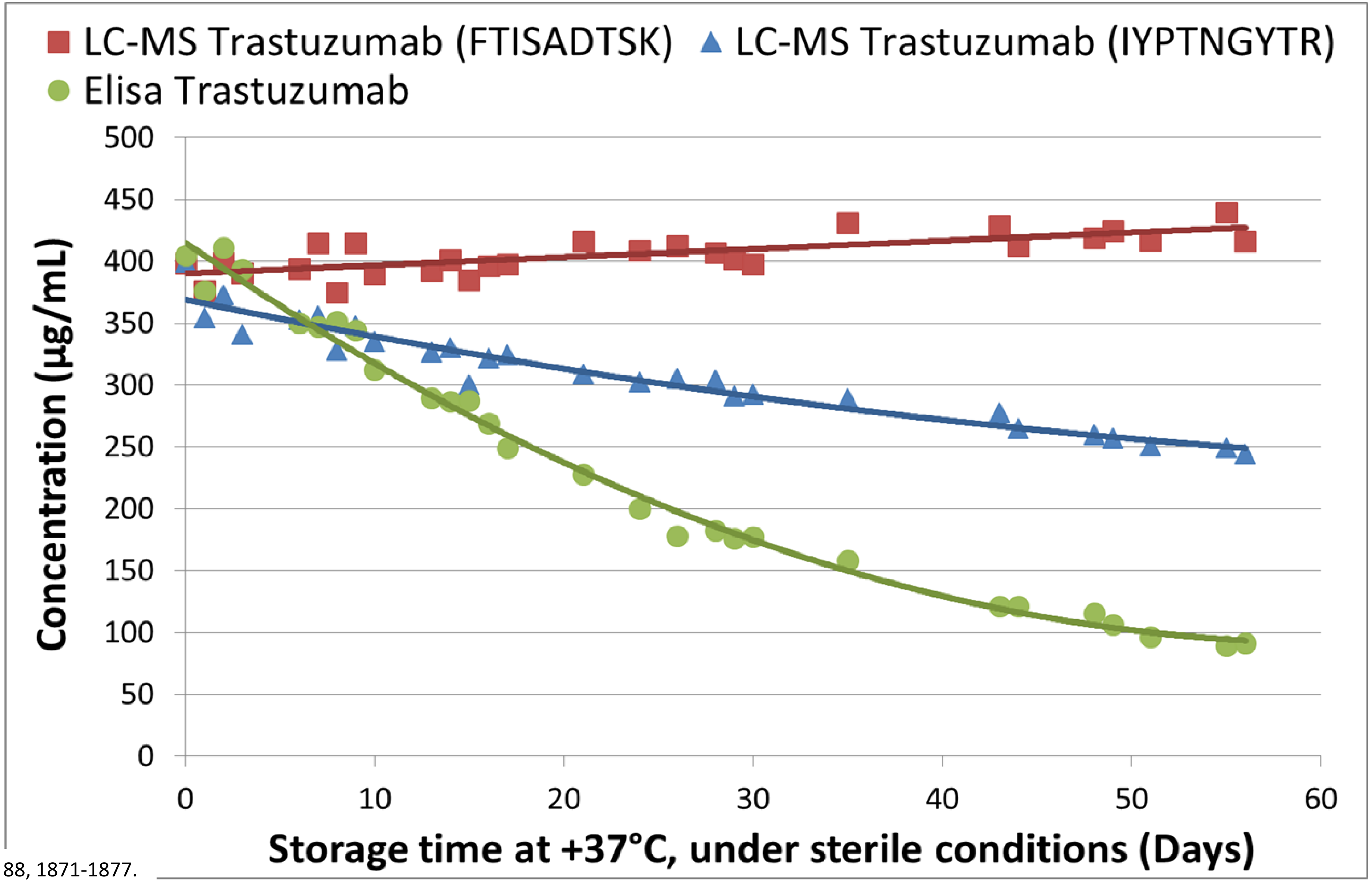
Bults et al., Anal Chem 2016, 88, 1871-1877.

Forced Stability Study in Plasma



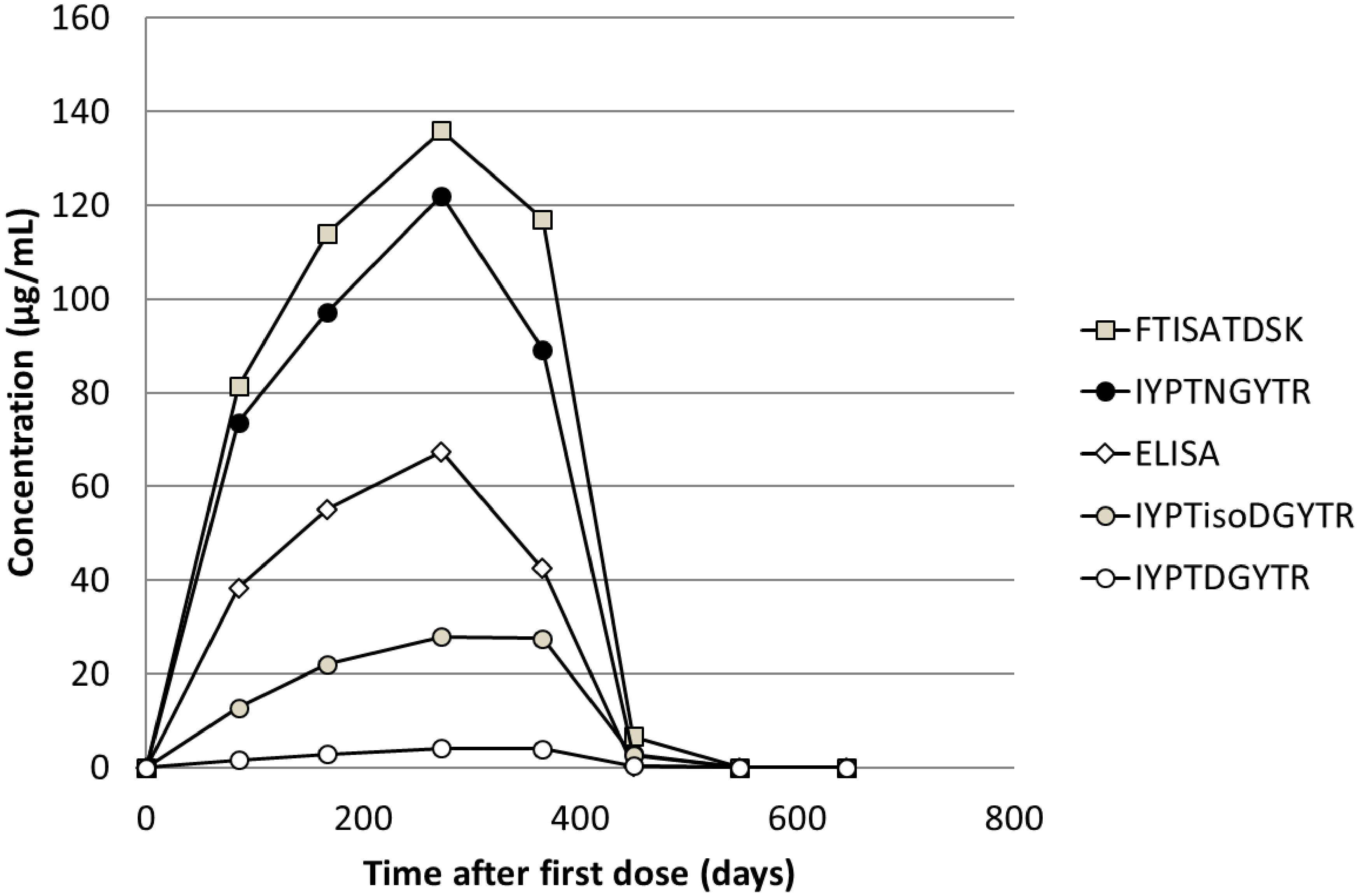
Bults et al., Anal Chem 2016, 88, 1871-1877.

Comparison LC-MS *vs.* ELISA



Bults et al., Anal Chem 2016, 88, 1871-1877.

In vivo Biotransformation



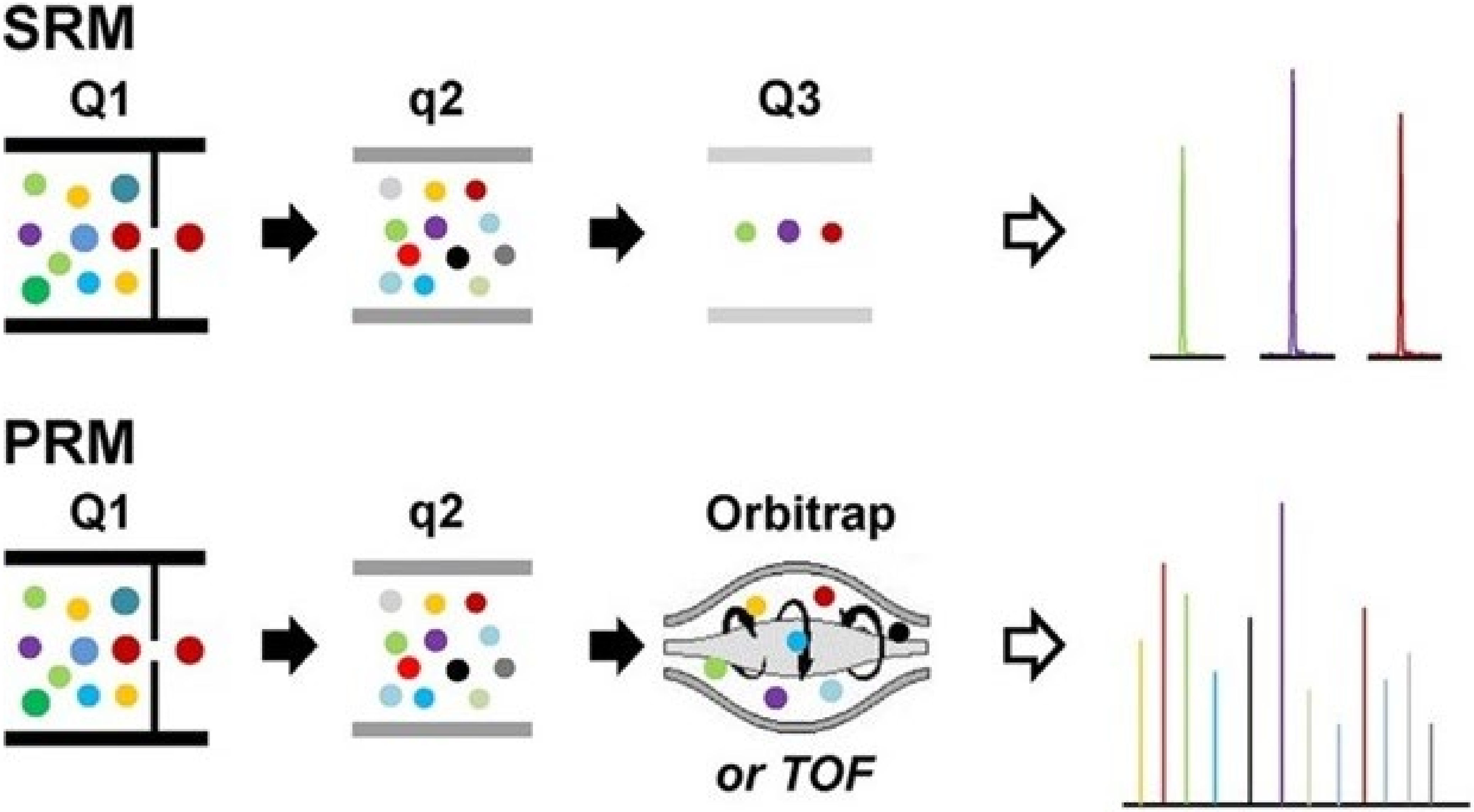
Bults et al., Anal Chem 2016, 88, 1871-1877.

Patient-Specific Deamidation

Subject	Sample Collection	ELISA	LC-MS	LC-MS	LC-MS	Ratio	Ratio
	Days after start of treatment		(FTISADTSK)	(IYPTNGYTR)	(IYPTisoDGYTR)	(total/isoD)	(N/isoD)
		(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)		
01	0	0	0	0	0	n.a.	n.a.
	86	20,1	43,9	34,5	4,50	9,8	7,7
	191	29,3	52,0	47,6	6,86	7,6	6,9
	296	**202**	**221**	**233**	**8,17**	**27,1**	**28,5**
	338	28,9	53,3	51,4	6,47	8,2	7,9
	505	0	0	0	0	n.a.	n.a.
	581	0	0	0	0	n.a.	n.a.
04	0	0	0	0	0	n.a.	n.a.
	77	36,9	105	85,2	24,0	4,4	3,6
	83	71,2	150	135	24,7	6,1	5,5
	146	60,6	152	126	31,4	4,8	4,0
	209	**210**	**371**	**353**	**45,1**	**8,2**	**7,8**
	357	79,6	263	197	67,0	3,9	2,9
	544	0,100	9,30	2,69	4,46	2,1	0,6
	678	0	0	0	0	n.a.	n.a.

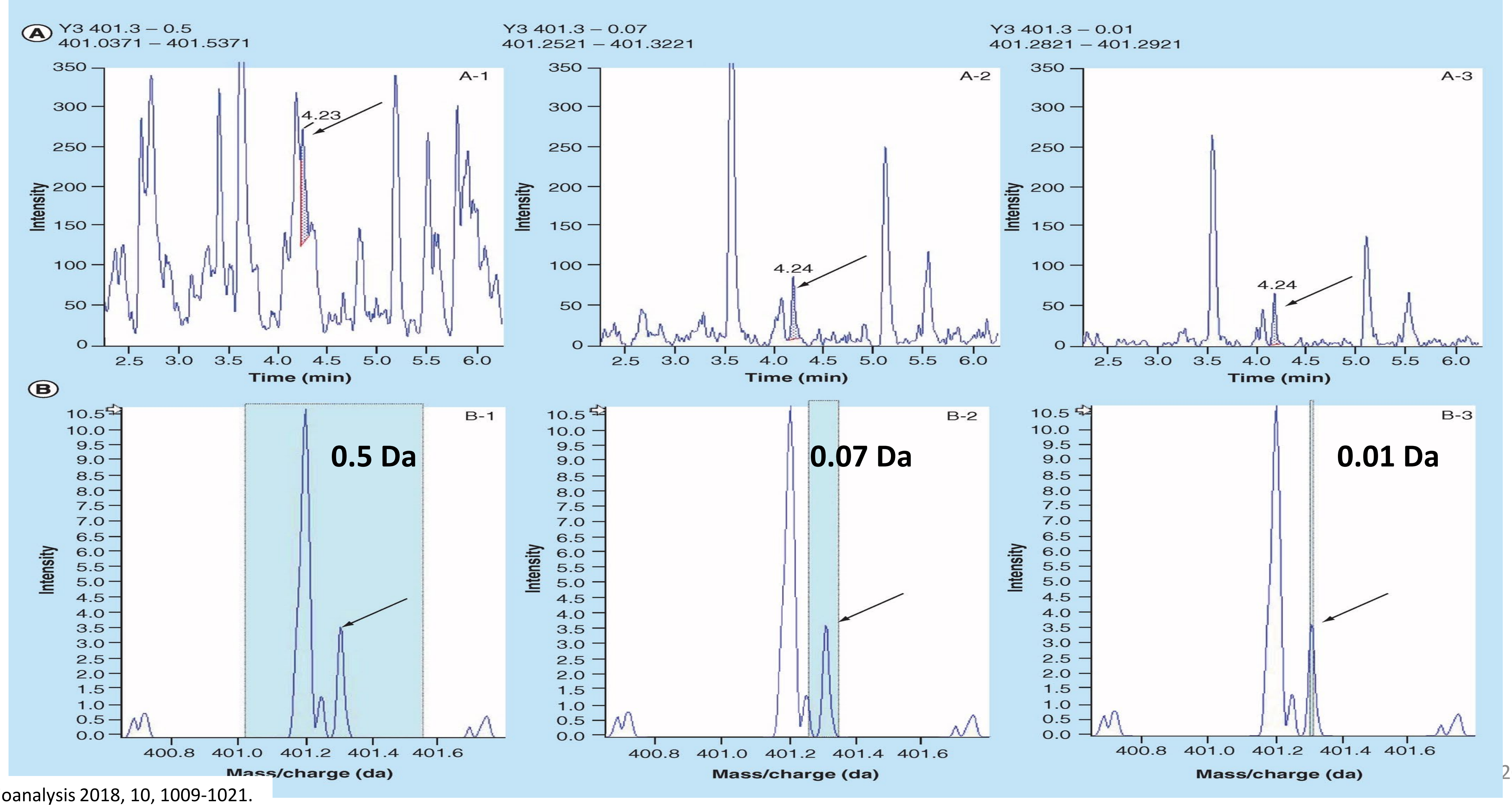
Bults et al., Anal Chem 2016, 88, 1871-1877.

LC-High-Resolution Mass Spectrometry in Protein Bioanalysis



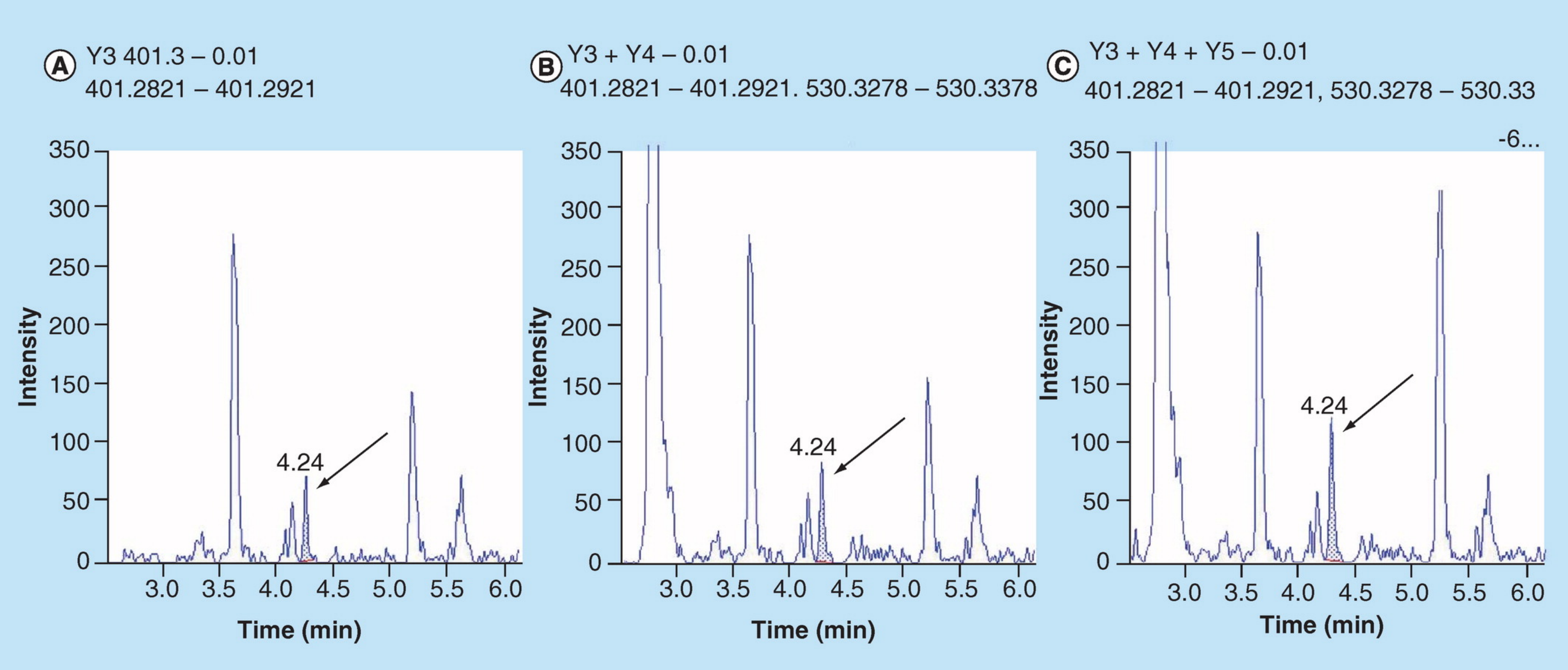
https://medicine.yale.edu/keck/nida/targetedproteomics/introduction.aspx

LC-High-Resolution MS: adapting the mass extraction window



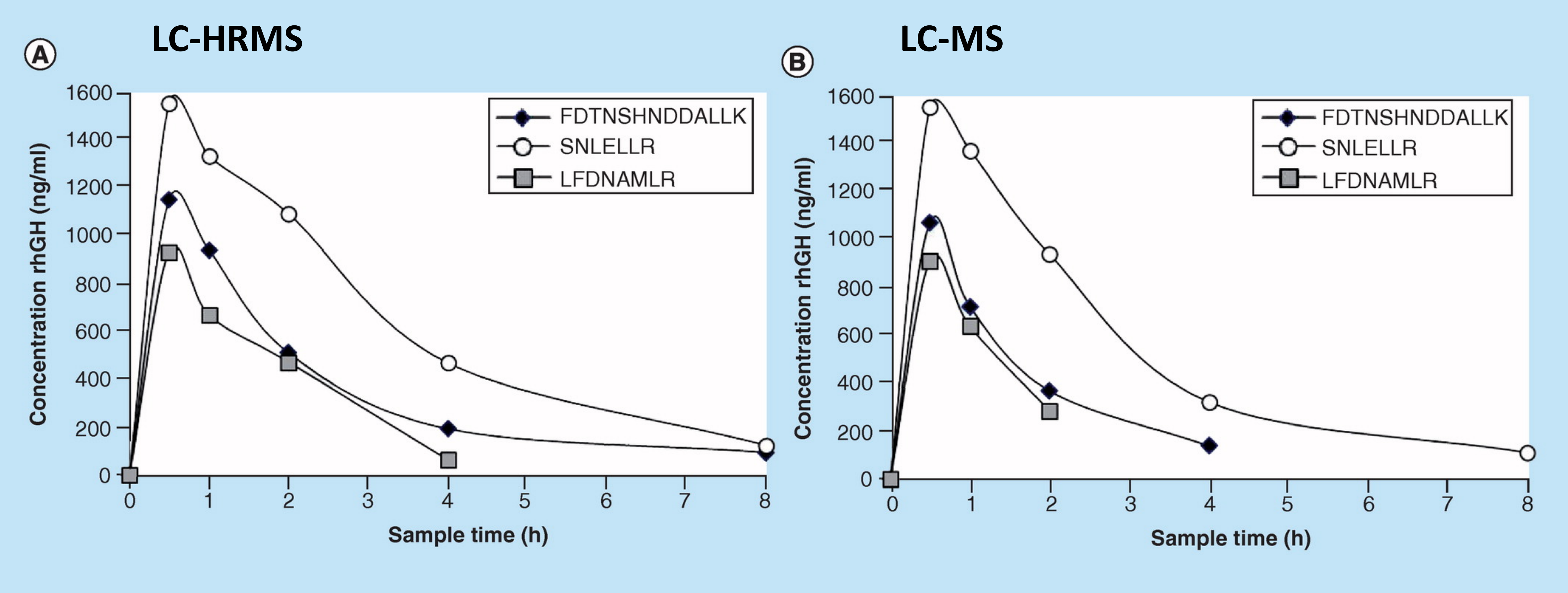
Bults et al., Bioanalysis 2018, 10, 1009-1021.

LC-High-Resolution MS: combining fragment ion traces



Bults et al., Bioanalysis 2018, 10, 1009-1021.

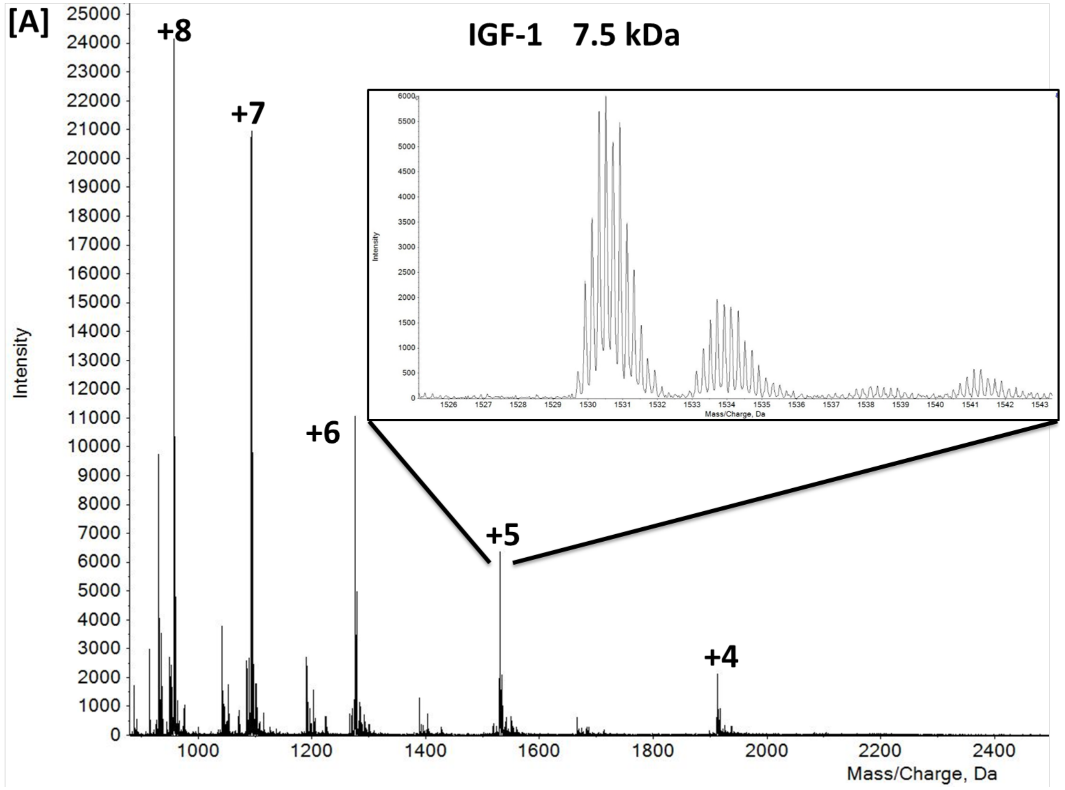
Comparison of LC-HRMS with LC-MS: quantification

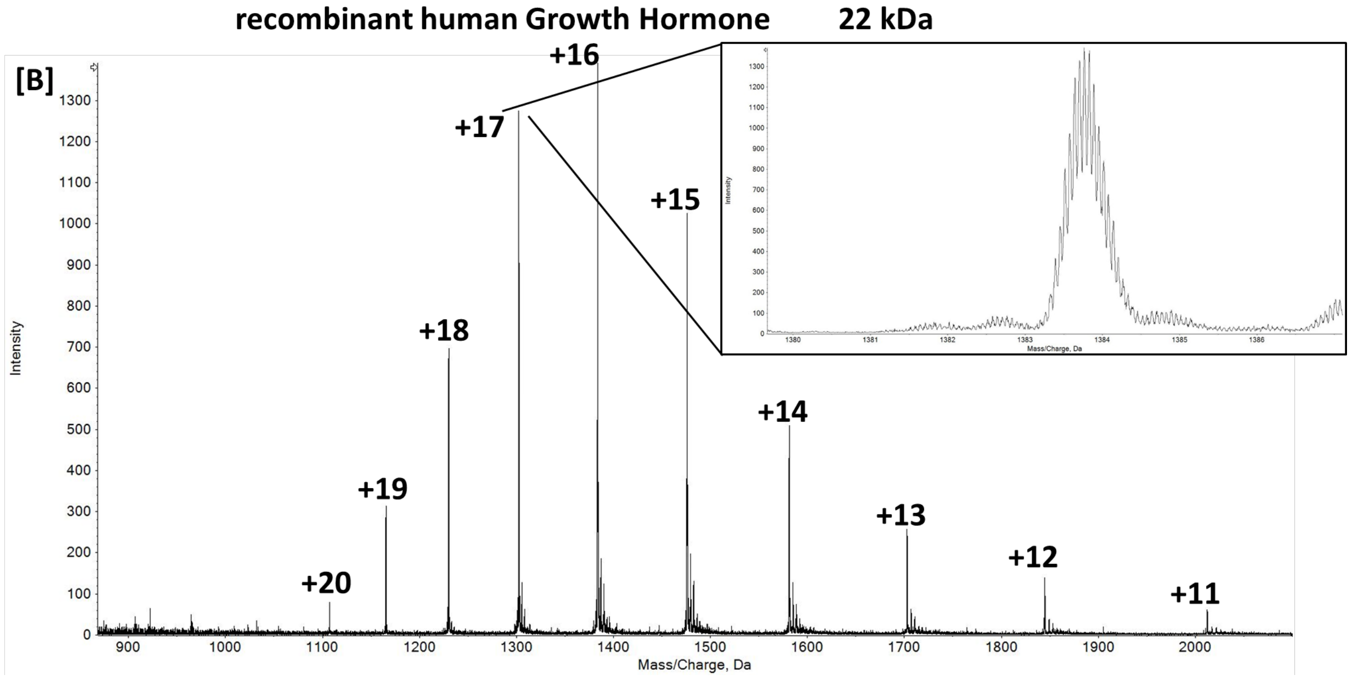


Bults et al., Bioanalysis 2018, 10, 1009-1021.

Bioanalysis at the Intact Protein Level (1)

Charge state envelope deconvolution and isotopologue resolution



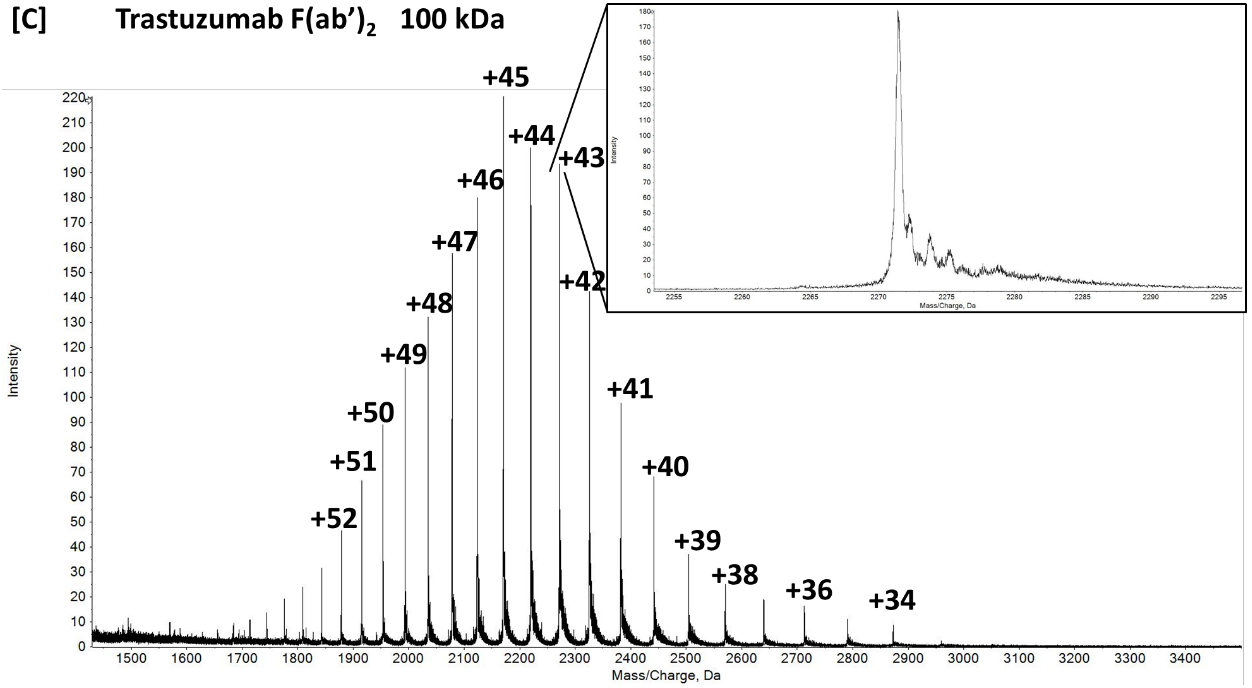


Bults et al., J. Chromatogr. B 2019, https://doi.org/10.1016/j.jchromb.2019.01.032

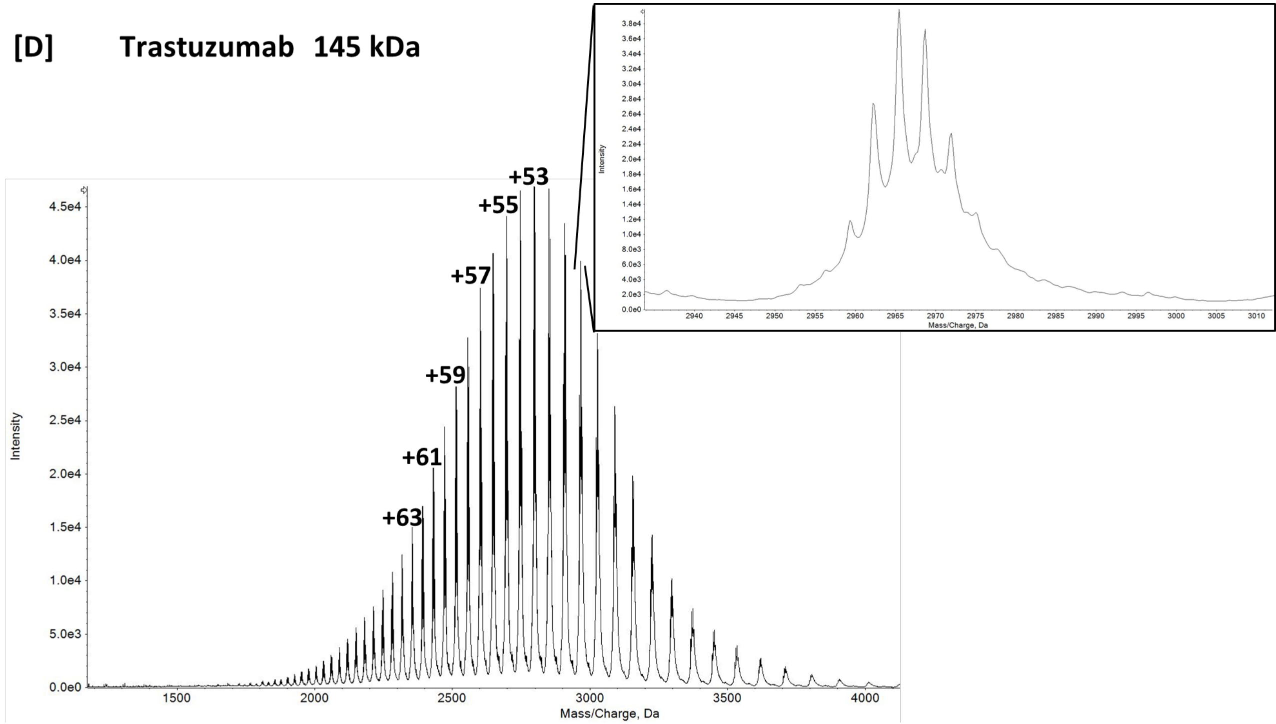
Bioanalysis at the Intact Protein Level (2)

Charge state envelope deconvolution and isotopologues resolution

[C] **Trastuzumab F(ab')$_2$ 100 kDa**



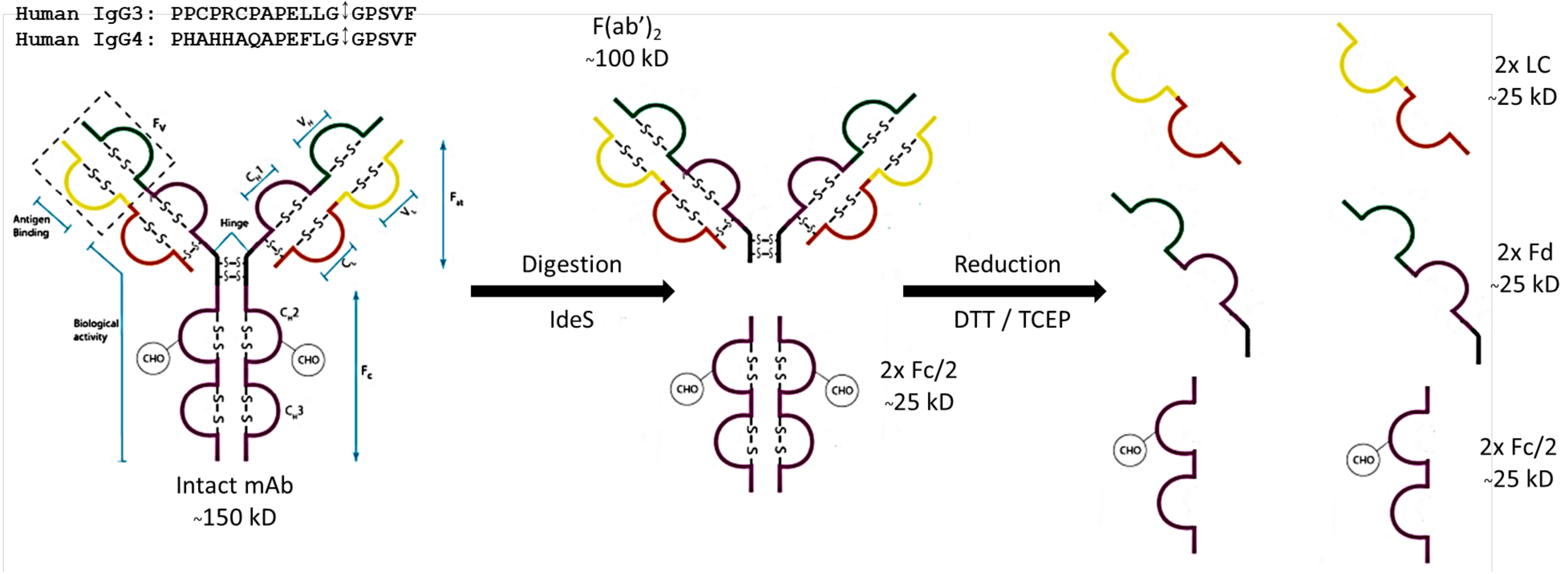
[D] **Trastuzumab 145 kDa**



Bults et al., J. Chromatogr. B 2019, https://doi.org/10.1016/j.jchromb.2019.01.032

Bioanalysis at the Protein Subunit Level

```
Human IgG1: HTCPPCPAPELLG↕GPSVF
Human IgG2: VECPPCPAPP_VA↕GPSVF
Human IgG3: PPCPRCPAPELLG↕GPSVF
Human IgG4: PHAHHAQAPEFLG↕GPSVF
```



Intact mAb
~150 kD

Digestion

IdeS

F(ab')$_2$
~100 kD

2x Fc/2
~25 kD

Reduction

DTT / TCEP

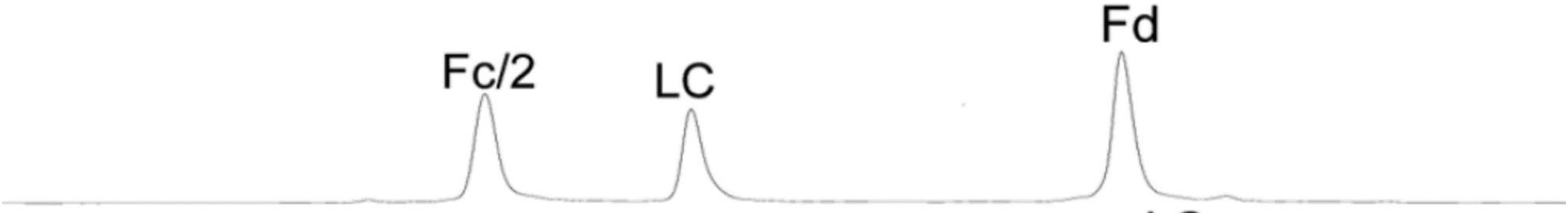
2x LC
~25 kD

2x Fd
~25 kD

2x Fc/2
~25 kD

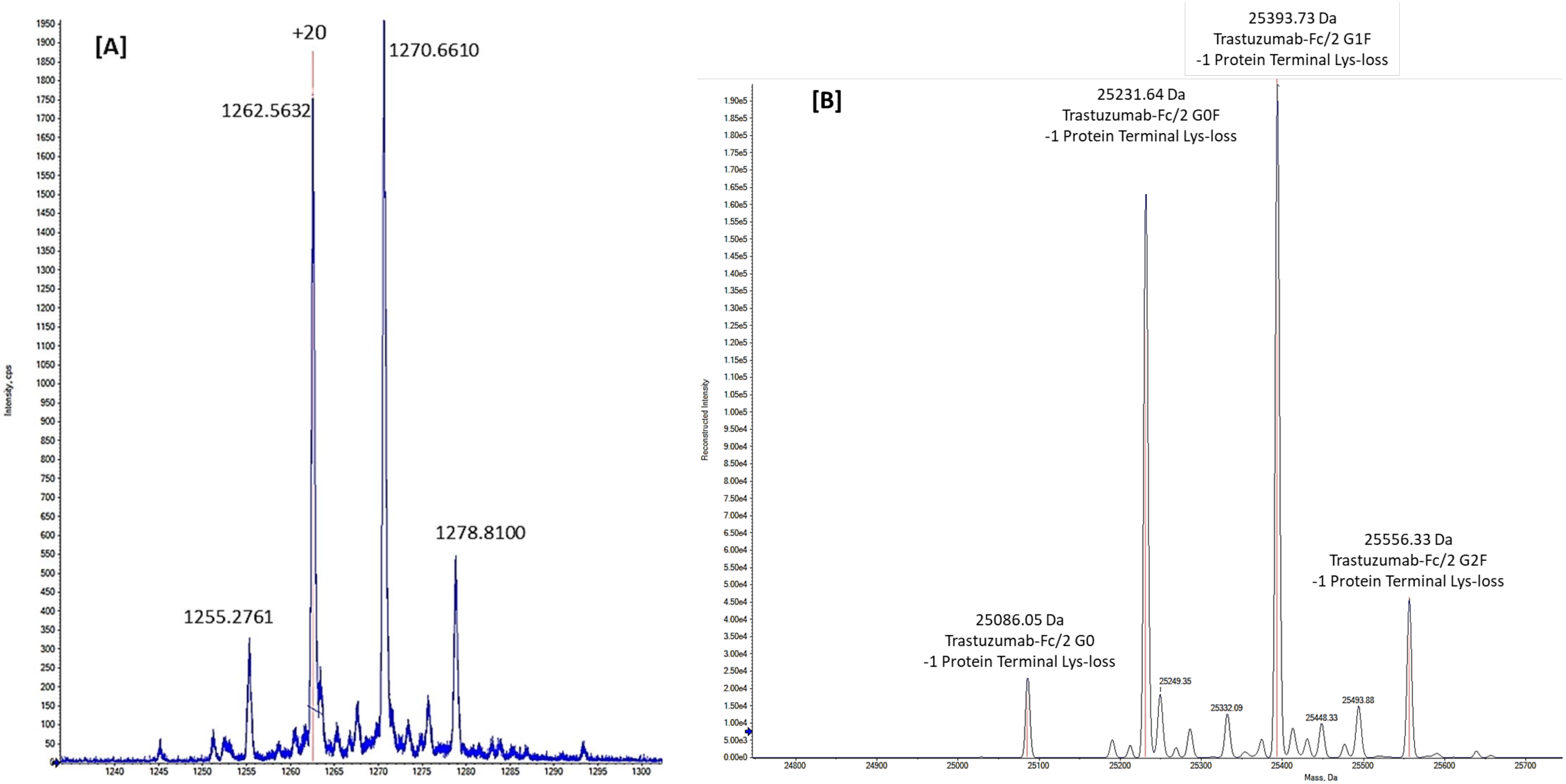
Bults et al., J. Chromatogr. B 2019, https://doi.org/10.1016/j.jchromb.2019.01.0

Bioanalysis at the Protein Subunit Level



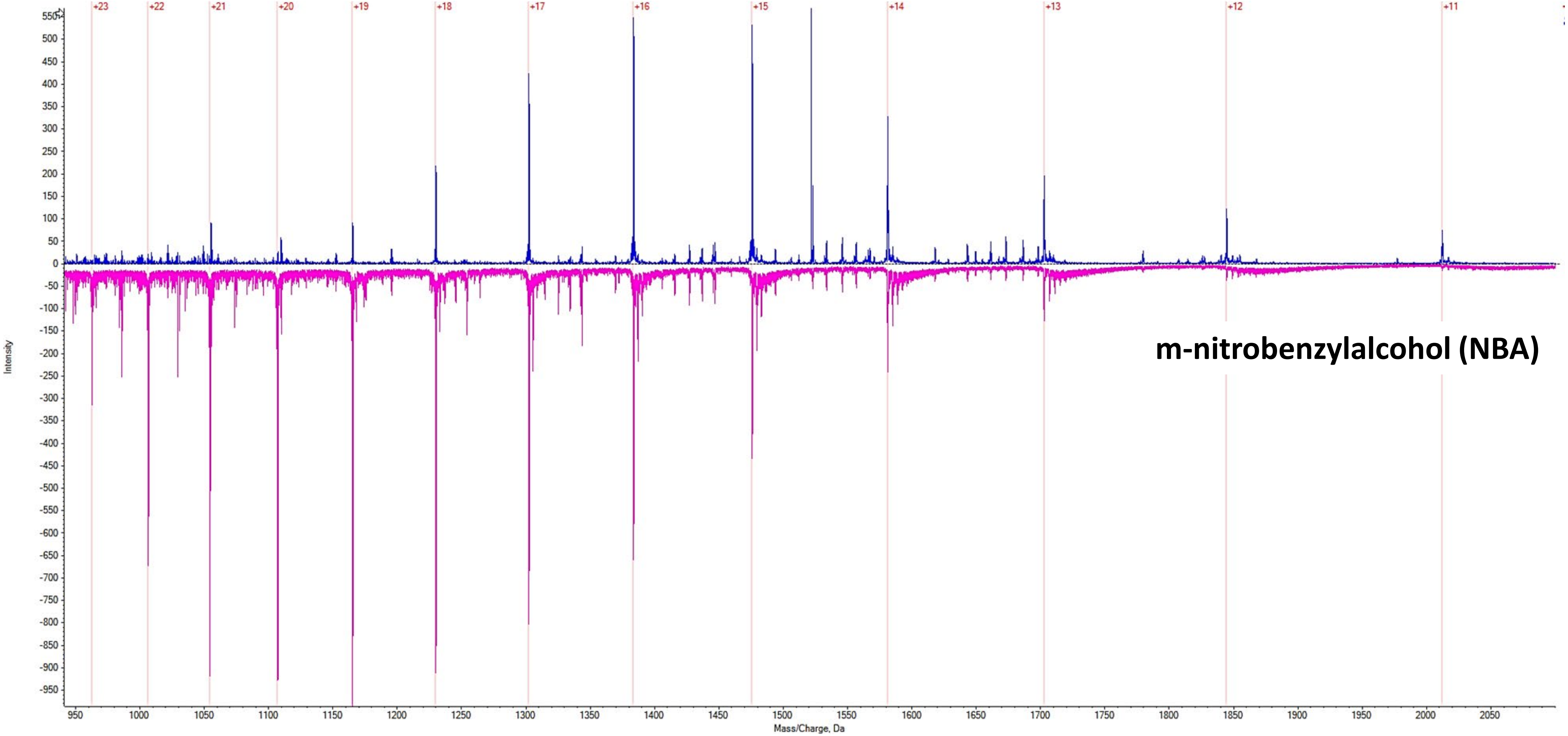
An et al., mAbs 2014, 6, 879-893.

Deconvolution of Charge State Envelope of the Fc/2 Fragment



Bults et al., J. Chromatogr. B 2019, https://doi.org/10.1016/j.jchromb.2019.01.032

Shifting the Charge-State Envelope - Supercharging

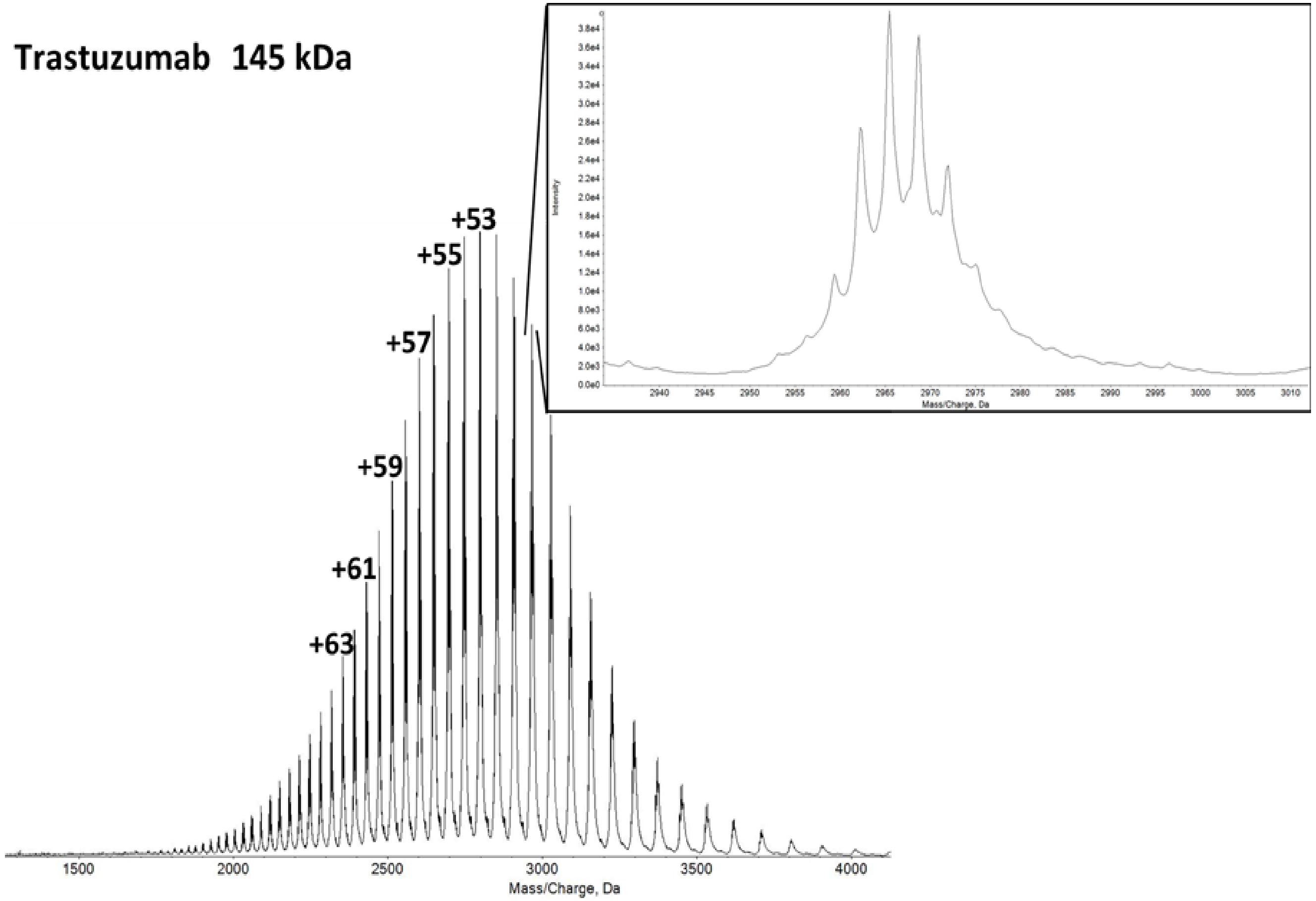


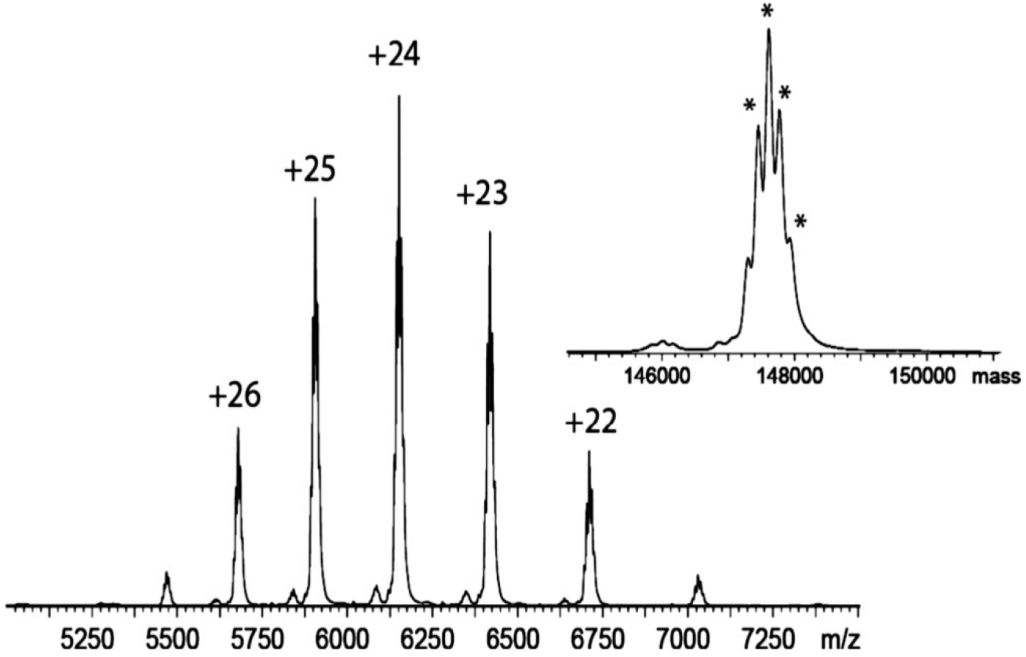
m-nitrobenzylalcohol (NBA)

Bults et al., J. Chromatogr. B 2019, https://doi.org/10.1016/j.jchromb.2019.01.032

Shifting the Charge-State Envelope – Native MS

Trastuzumab 145 kDa





Bults et al., J. Chromatogr. B 2019, https://doi.org/10.1016/j.jchromb.2019.01.032
Thompson et al., Methods 2014, 65, 11-17.

Targeted Biomarker Analysis by LC-MS at the pM Level

Chronic Obstructive Pulmonary Disease (COPD)

GLOBAL INITIATIVE FOR CHRONIC OBSTRUCTIVE LUNG DISEASE™

- COPD, a common preventable disease, is characterized by **persistent airflow limitation** that is usually progressive and associated with an enhanced chronic **inflammatory response** in the airways and the lung **to noxious particles or gases**
- **Exacerbations and comorbidities** contribute to the overall severity in individual patients.

Phenotypes of COPD

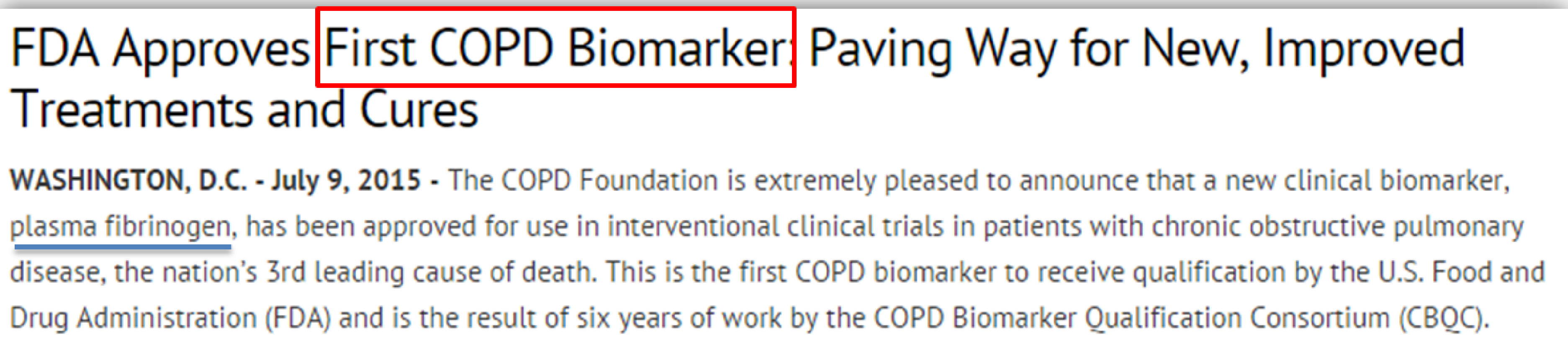
Pink Puffer

Blue Bloater

Need for COPD Biomarkers

- Susceptibility to develop COPD
- Decline in FEV1
- Corticosteroid response
- Risk of Exacerbations

COPDfoundation.org:



FDA Approves First COPD Biomarker: Paving Way for New, Improved Treatments and Cures

WASHINGTON, D.C. - July 9, 2015 - The COPD Foundation is extremely pleased to announce that a new clinical biomarker, plasma fibrinogen, has been approved for use in interventional clinical trials in patients with chronic obstructive pulmonary disease, the nation's 3rd leading cause of death. This is the first COPD biomarker to receive qualification by the U.S. Food and Drug Administration (FDA) and is the result of six years of work by the COPD Biomarker Qualification Consortium (CBQC).

How to Prioritize a Biomarker for COPD?

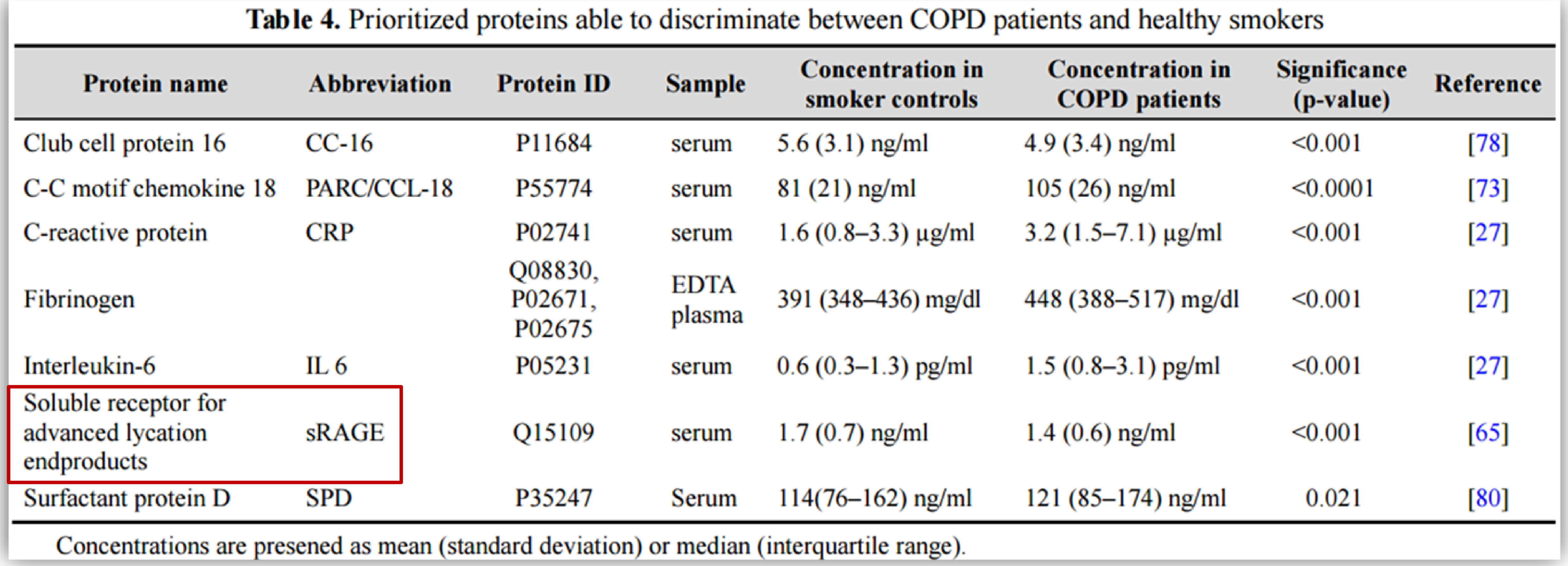


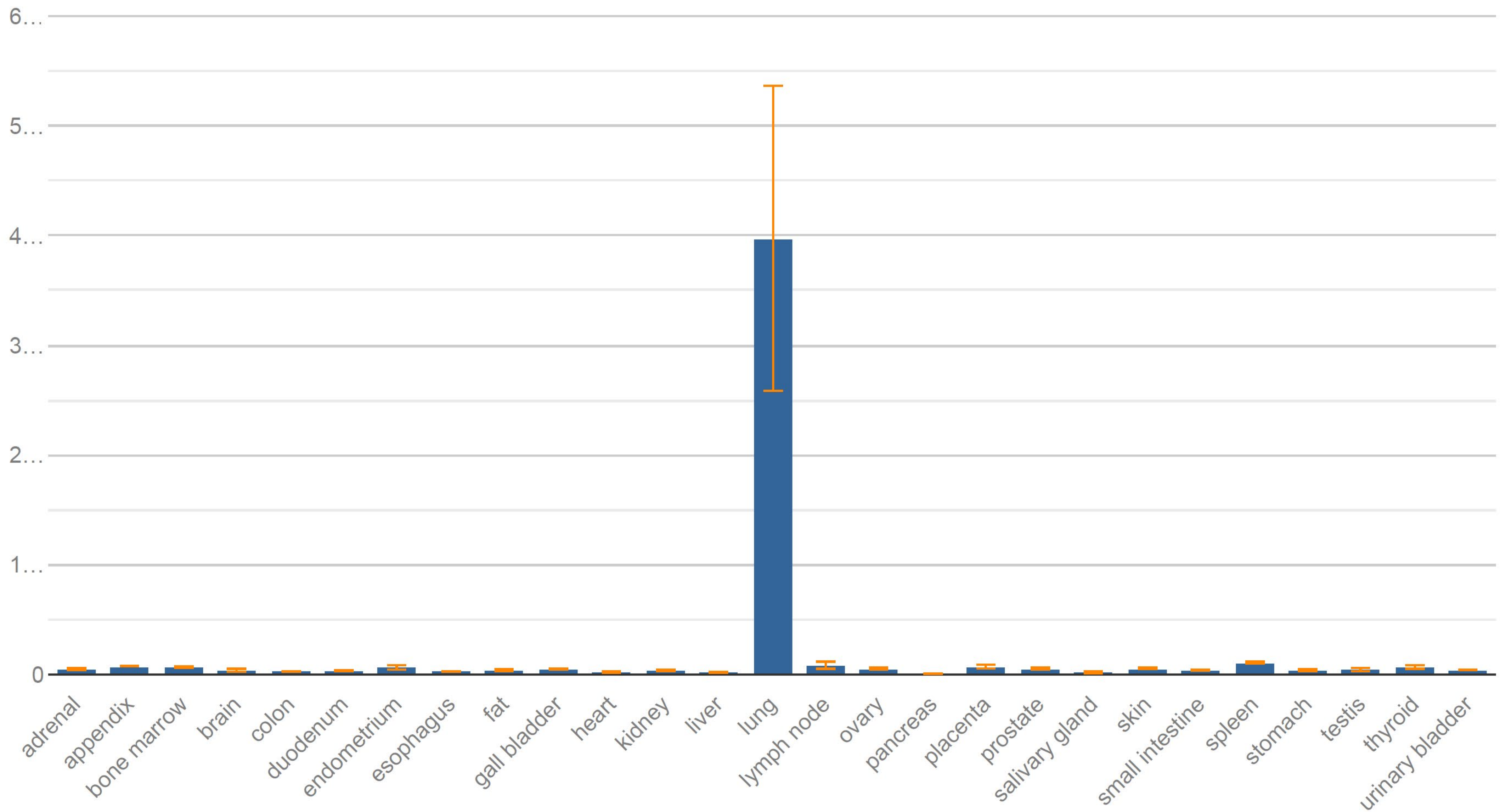
Table 4. Prioritized proteins able to discriminate between COPD patients and healthy smokers

Protein name	Abbreviation	Protein ID	Sample	Concentration in smoker controls	Concentration in COPD patients	Significance (p-value)	Reference
Club cell protein 16	CC-16	P11684	serum	5.6 (3.1) ng/ml	4.9 (3.4) ng/ml	<0.001	[78]
C-C motif chemokine 18	PARC/CCL-18	P55774	serum	81 (21) ng/ml	105 (26) ng/ml	<0.0001	[73]
C-reactive protein	CRP	P02741	serum	1.6 (0.8–3.3) μg/ml	3.2 (1.5–7.1) μg/ml	<0.001	[27]
Fibrinogen		Q08830, P02671, P02675	EDTA plasma	391 (348–436) mg/dl	448 (388–517) mg/dl	<0.001	[27]
Interleukin-6	IL 6	P05231	serum	0.6 (0.3–1.3) pg/ml	1.5 (0.8–3.1) pg/ml	<0.001	[27]
Soluble receptor for advanced lycation endproducts	sRAGE	Q15109	serum	1.7 (0.7) ng/ml	1.4 (0.6) ng/ml	<0.001	[65]
Surfactant protein D	SPD	P35247	Serum	114(76–162) ng/ml	121 (85–174) ng/ml	0.021	[80]

Concentrations are presened as mean (standard deviation) or median (interquartile range).

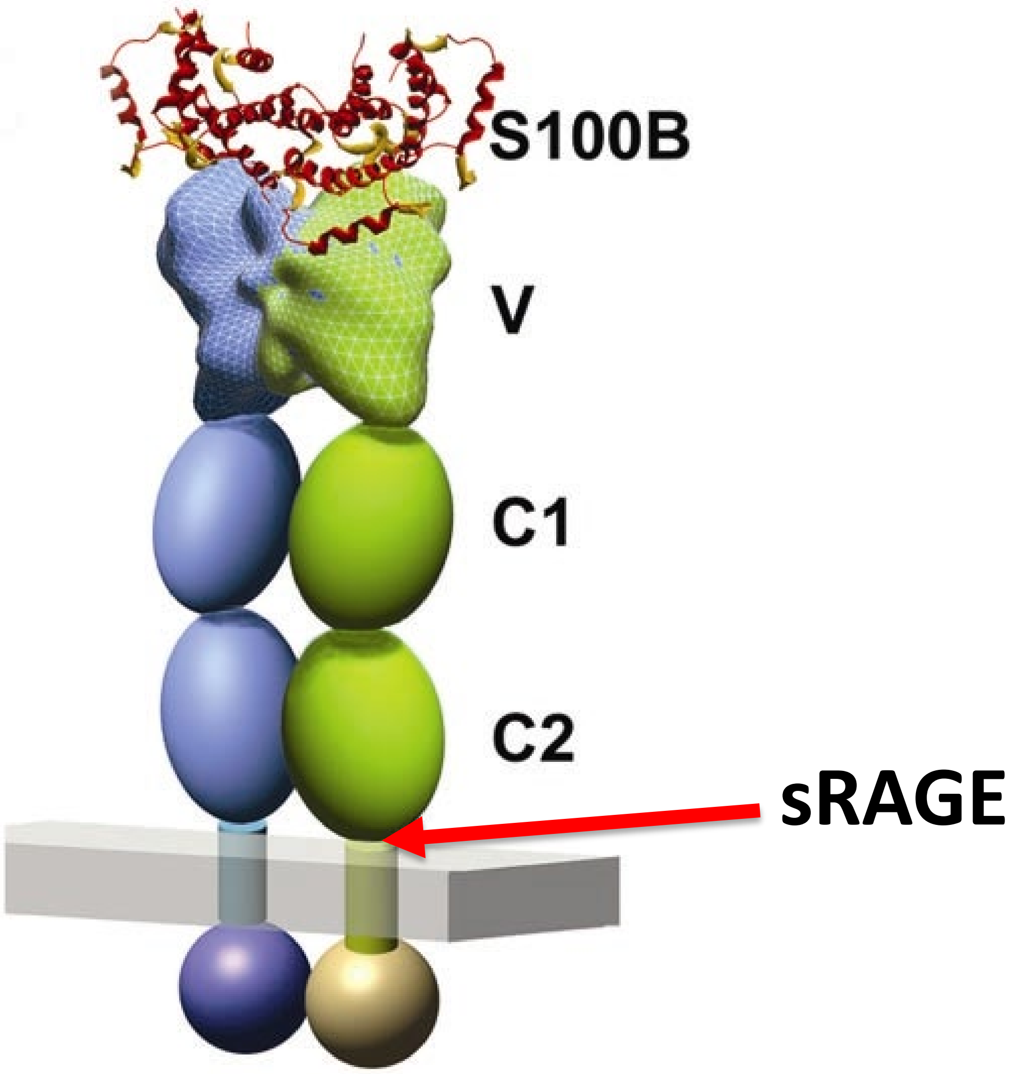
Ongay et al., Advances in Precision Medicine 2016, 1, 12-24.

Expression of the AGER Gene

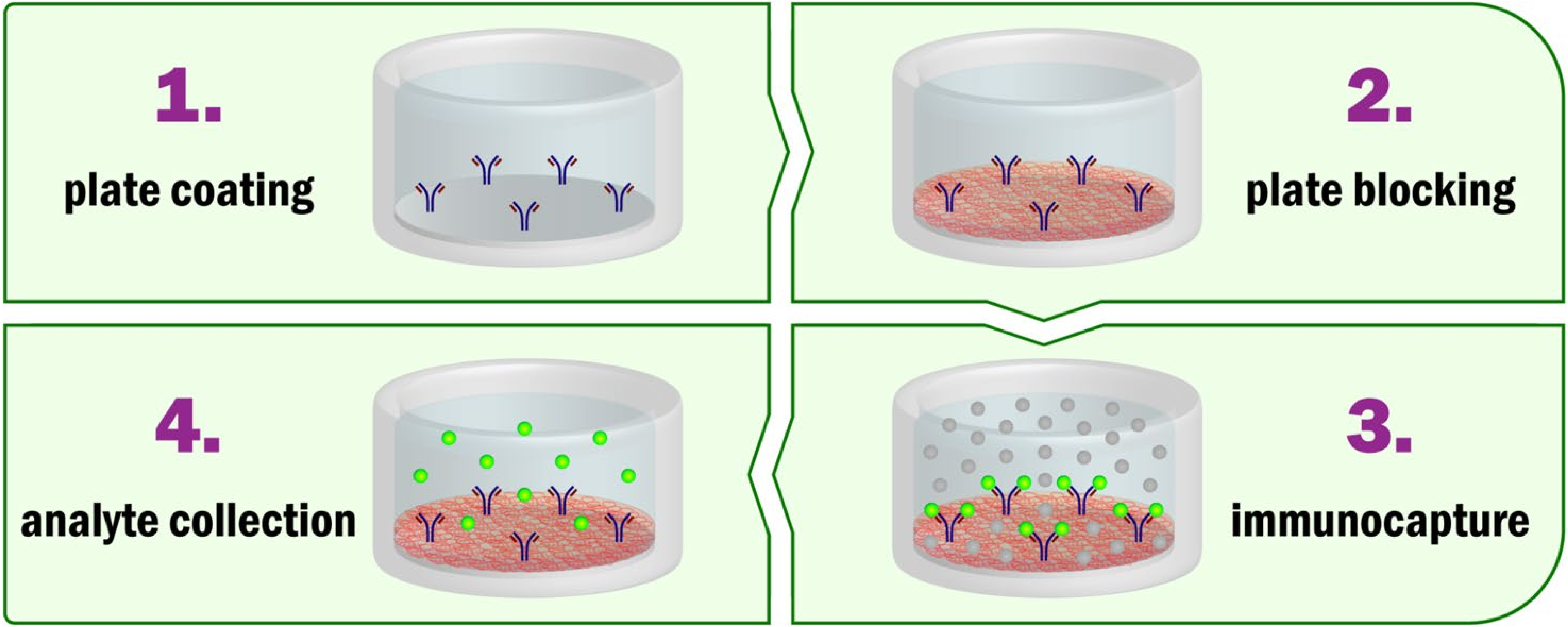


Fagerberg et al., Molecular & Cellular Proteomics 2014, 13, 397-406.

Receptor for Advanced Glycation Endproducts (RAGE)

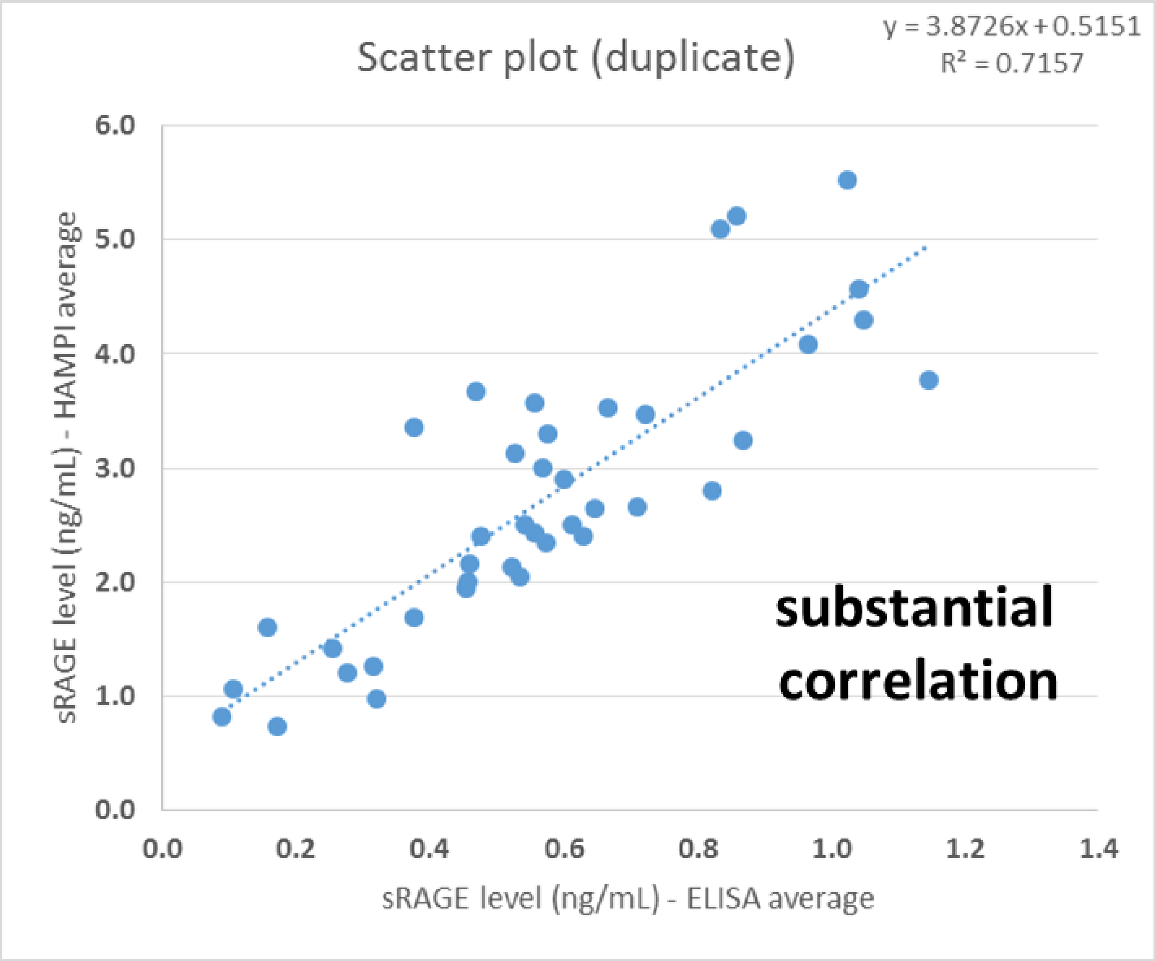


Enrichment of sRAGE from Serum



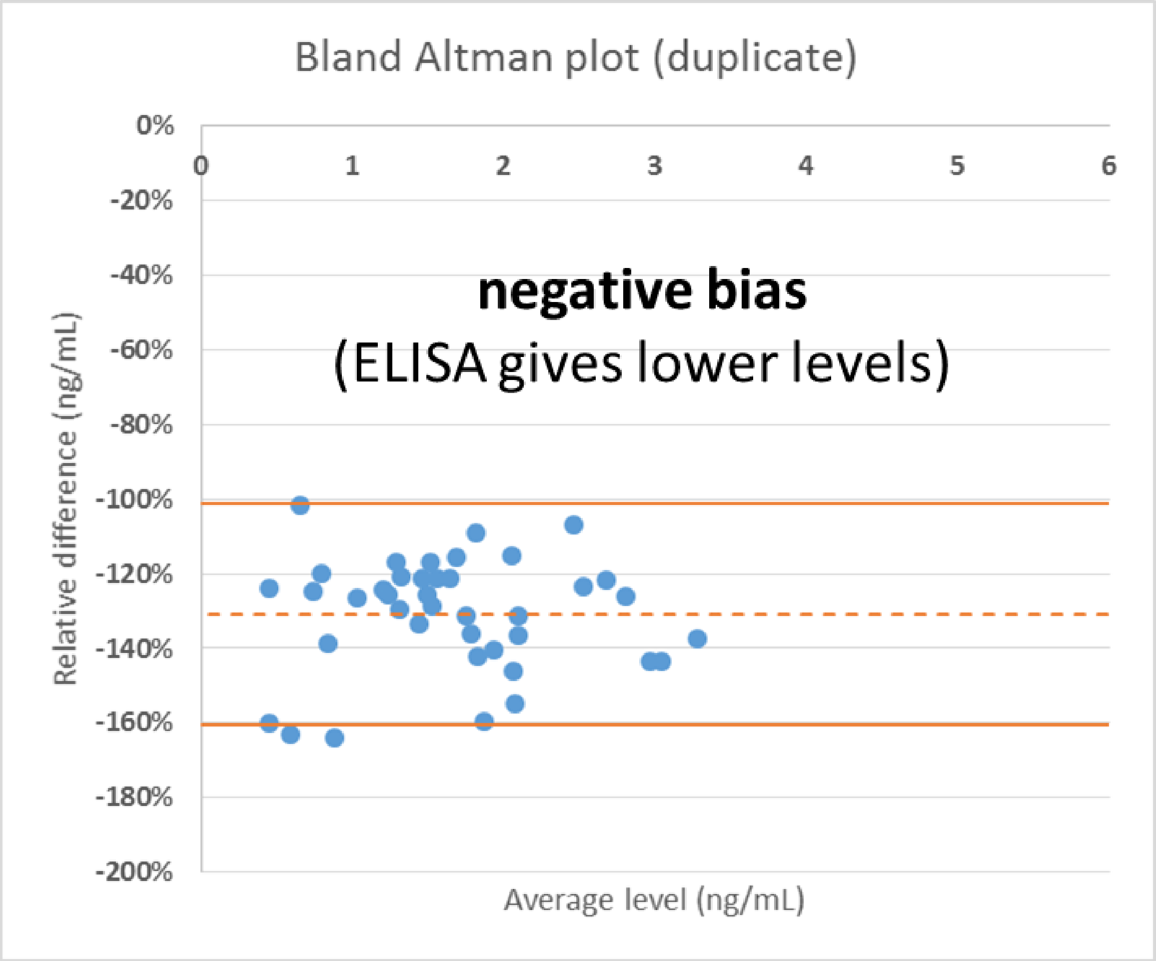
Klont et al., *Talanta*, *182*, **2018**, 414-421.

Comparison: Immunoaffinity LC-MS *vs.* ELISA



Scatter plot (duplicate)

substantial correlation



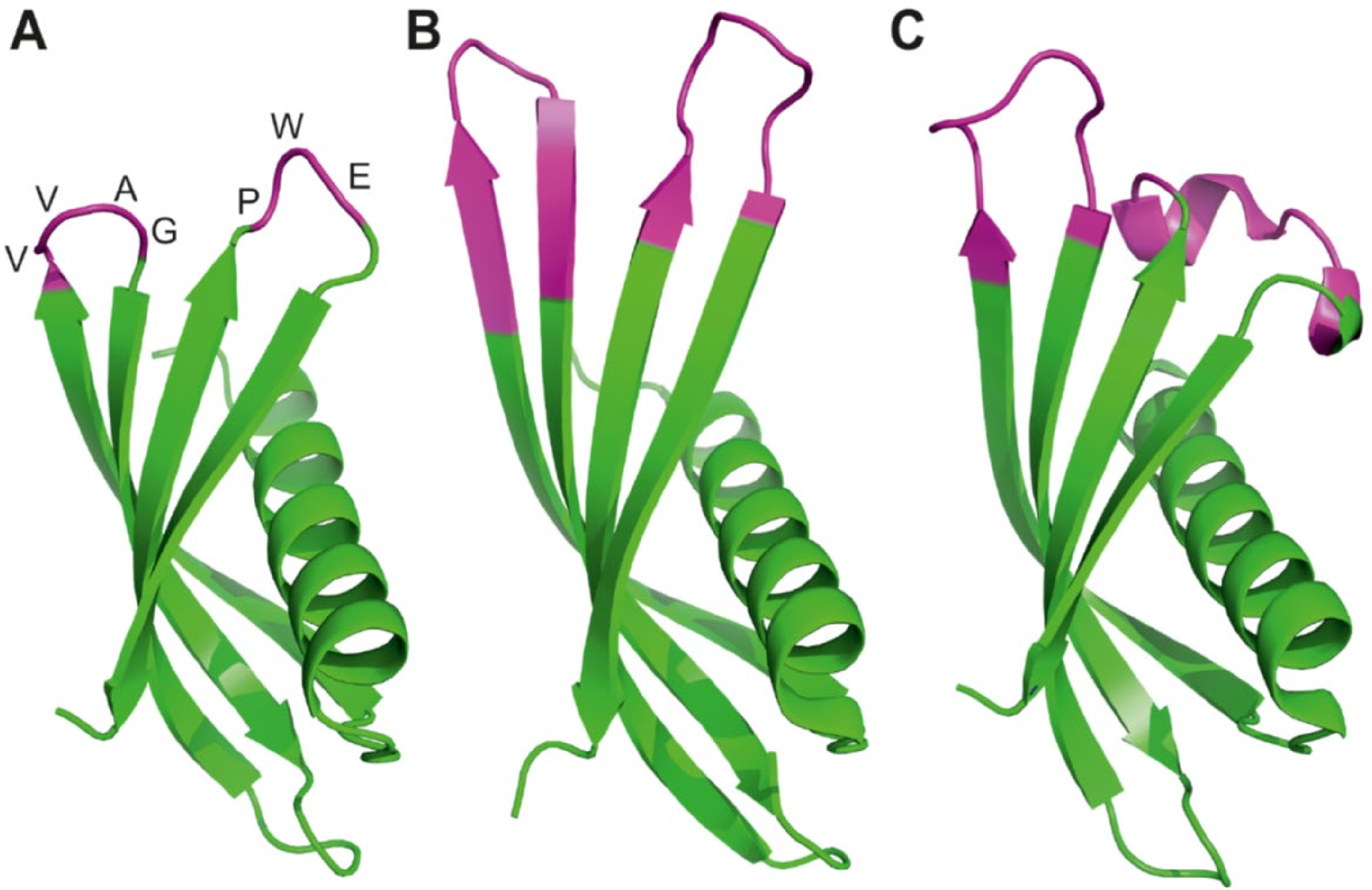
Bland Altman plot (duplicate)

negative bias
(ELISA gives lower levels)

→ Lower levels by ELISA are due to an insufficient amount of primary antibody

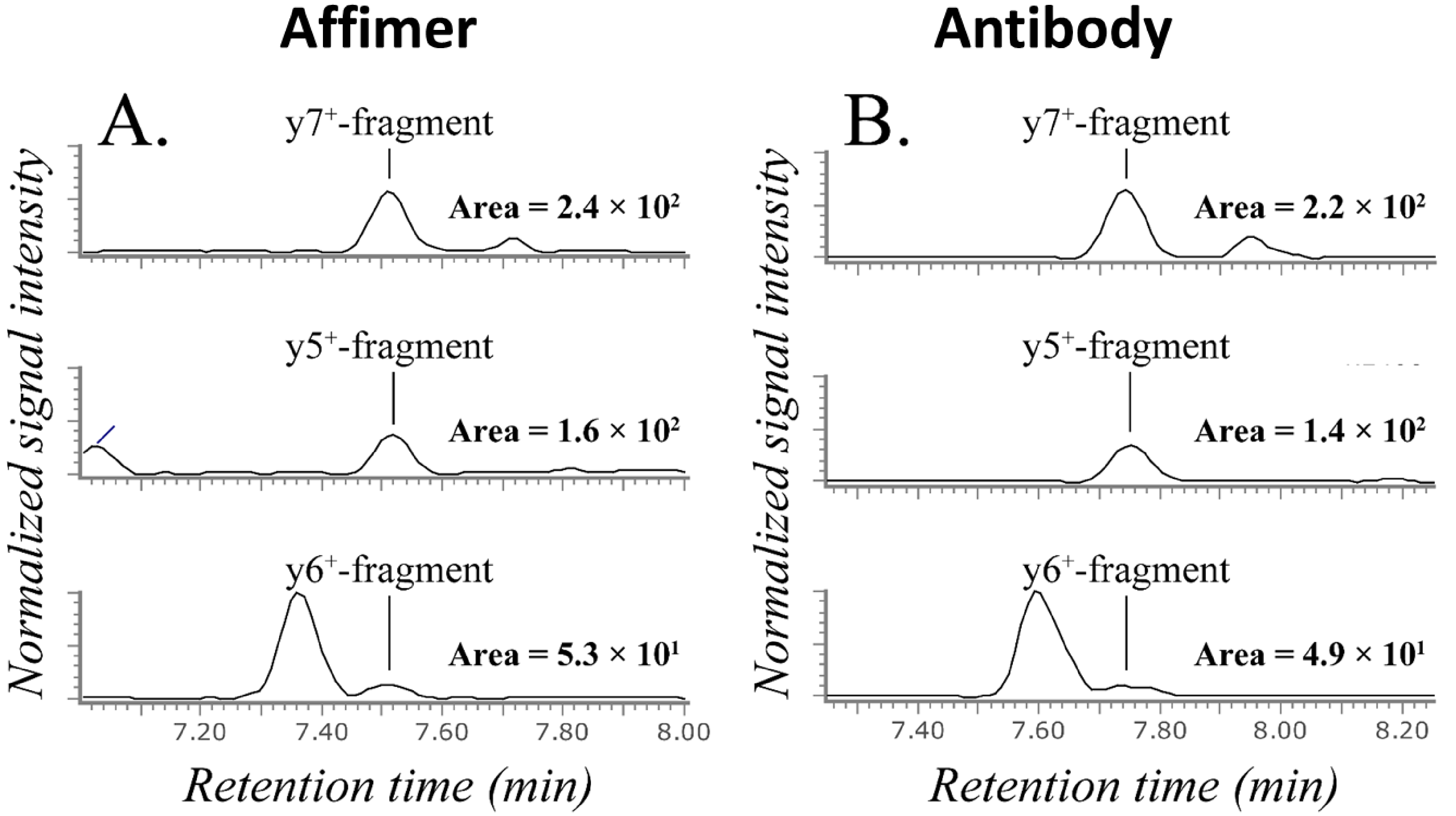
Klont et al., *Talanta*, *182*, **2018**, 414-421.

Alternative Affinity Agents: Affimers



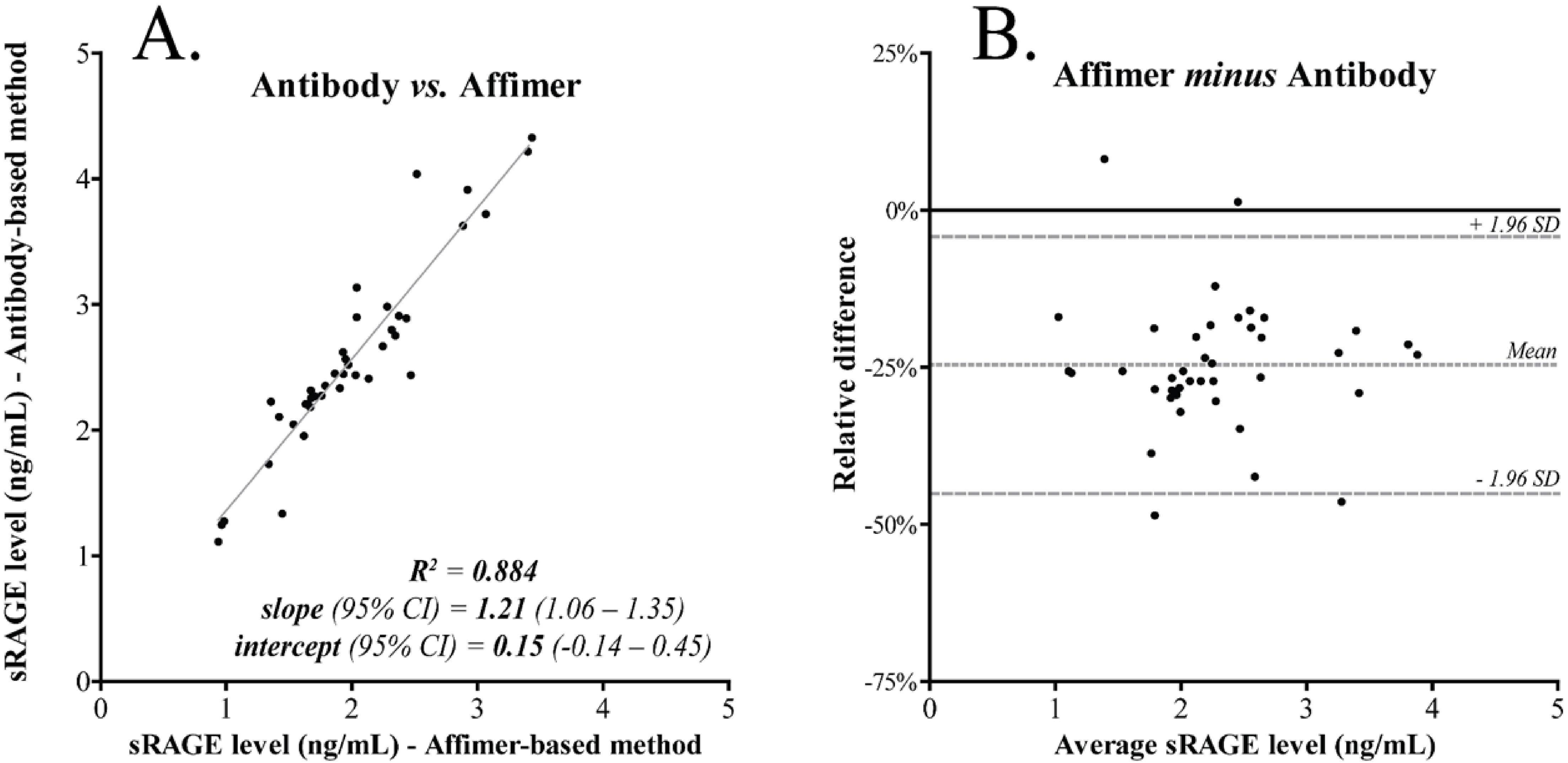
Tiede et al., *Elife* **2017**, 6, e24903.

Affimer-Based Enrichment *vs.* Antibody-Based Enrichment (1)



Klont et al., *J. Proteome Res., 8,* **2018**, 2892-2899

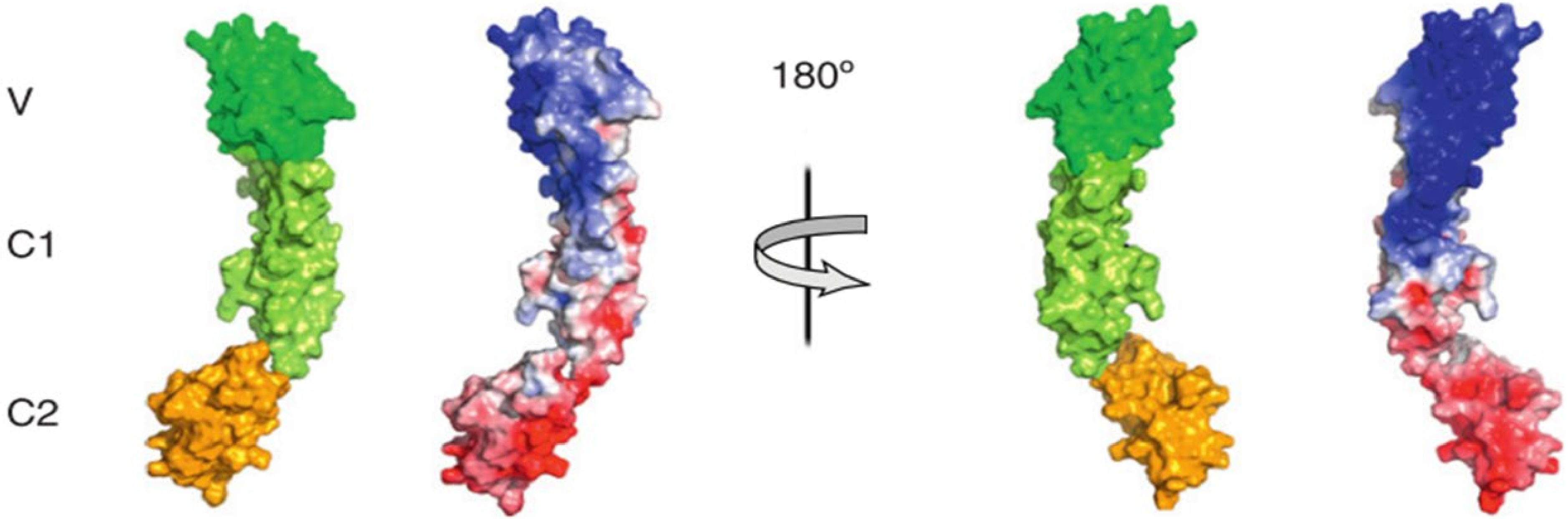
Affimer-based Enrichment *vs.* Antibody-based Enrichment (2)



Klont et al., *J. Proteome Res., 8,* **2018**, 2892-2899

sRAGE Enrichment Without Affinity Ligands

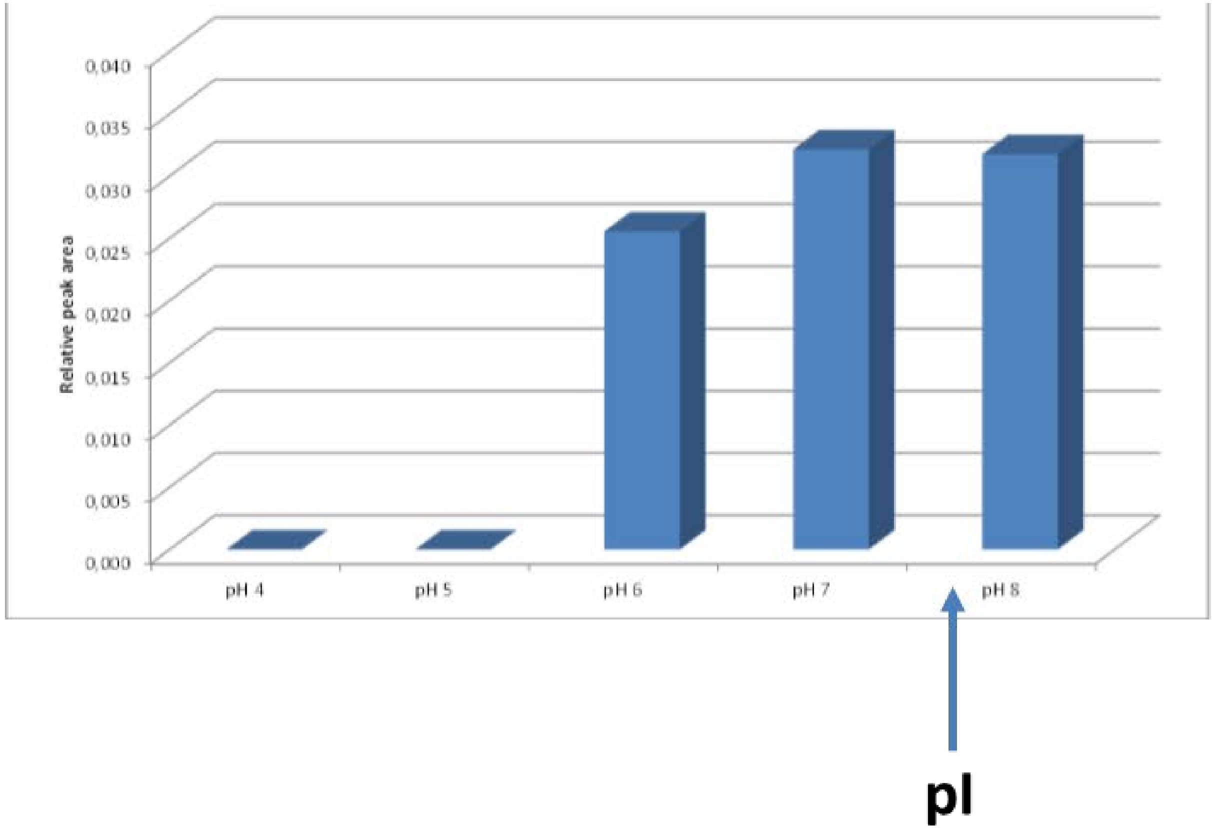
- **Calculated pI: 7.81**
- **Charge distribution**
- **V/C1 tandem domain is rich in arginine and lysine**



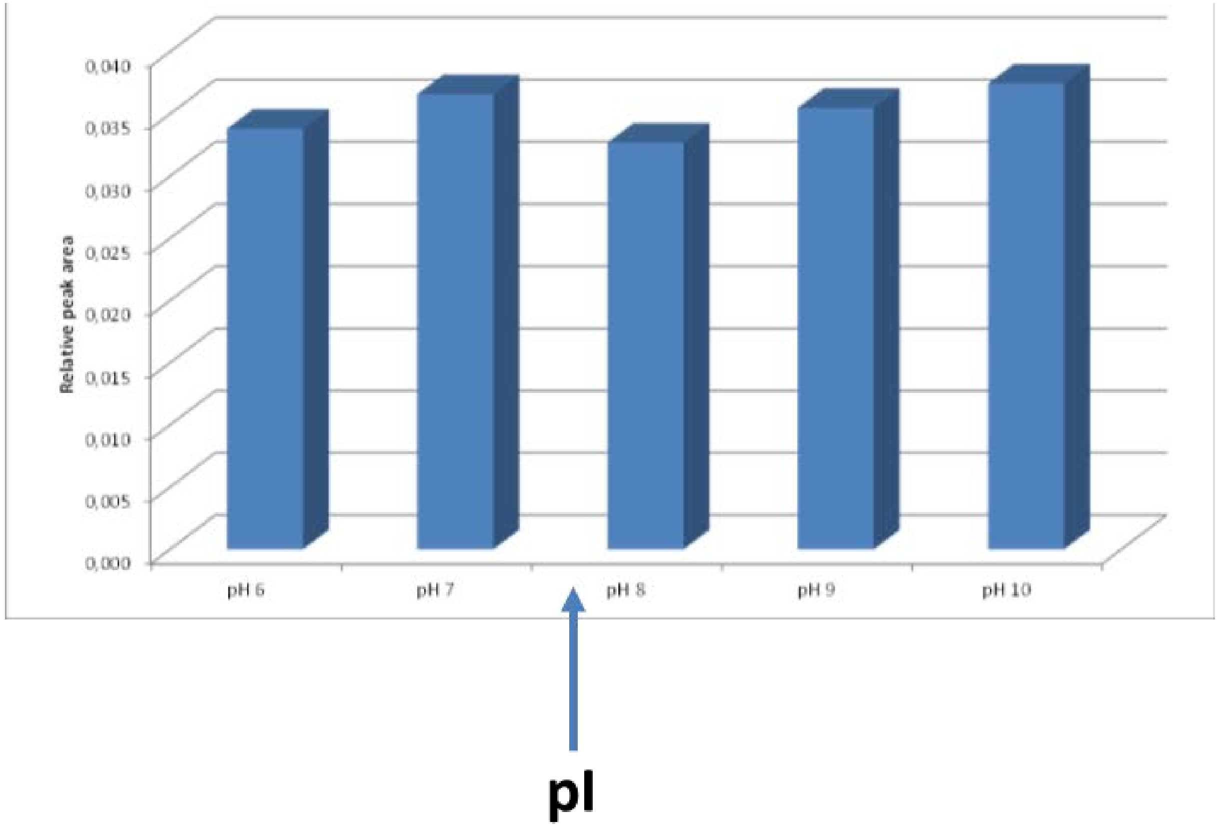
Fritz, Trends Biochem Sci. 2011;36, 625-632.

Enrichment of sRAGE by Ion-Exchange SPE

Strong Anion-Exchange



Strong Cation-Exchange



Klont et al., Anal. Chim. Acta, **2018**, 1043, 45-51

SCX-SPE at pH 10

Strong Cation Exchange (SCX)

- Condition with H_2O
- **Load samples with 20 mM ammonium acetate, pH 10**
- **Wash with 20 mM ammonium acetate, pH 10**
- **Elute with 1% ammonia** → Collect in 15% CH_3COOH

Evaporate eluent → Add SIL-peptides in digestion buffer (100 mM ABC)

Tryptic digestion

- Reduction (DTT)
- Alkylation (IAM)
- IAM quenching (DTT)
- Digestion (Overnight)

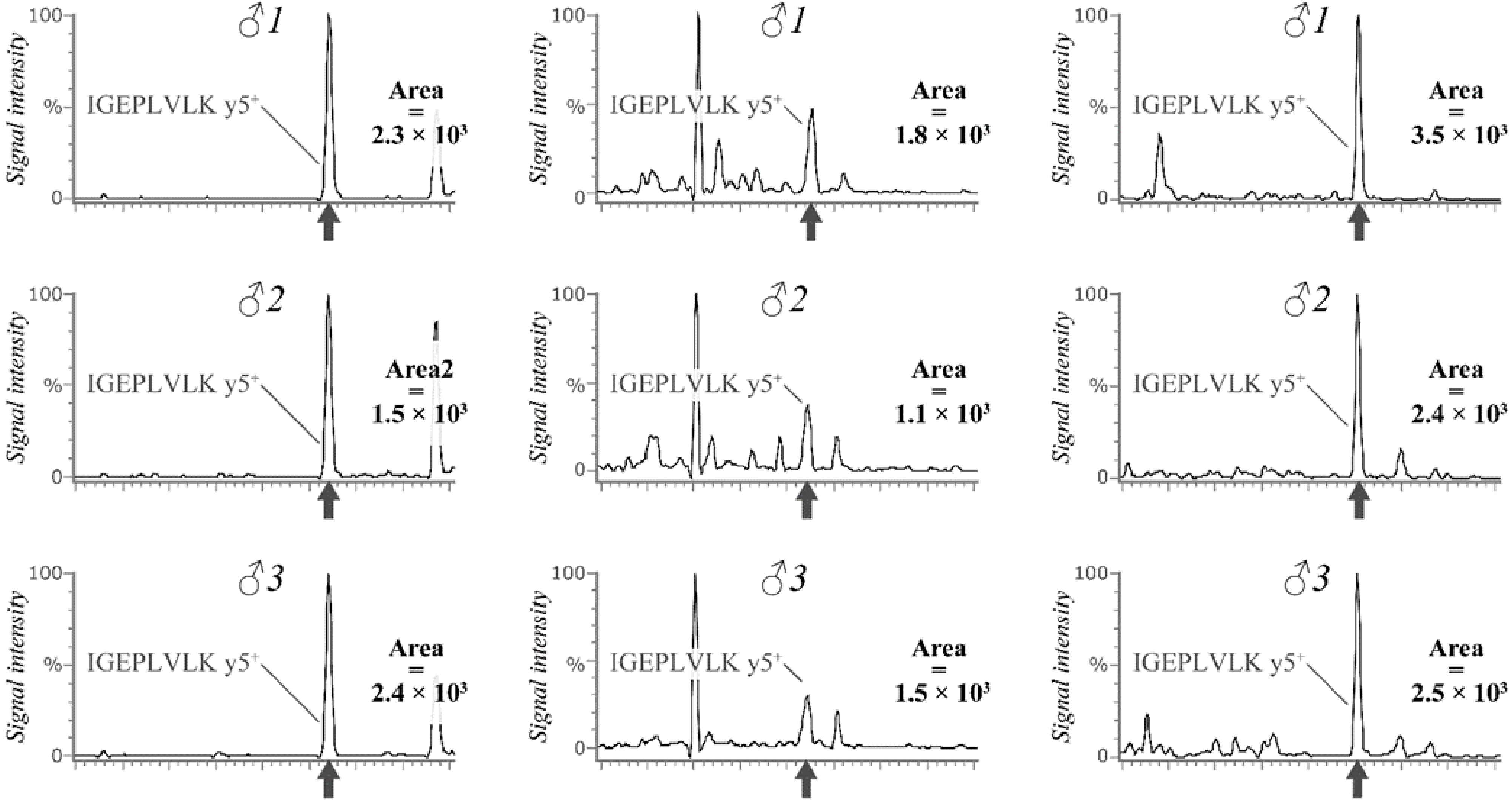
LC-MS/MS analysis

- IGEPLVLK ($y5^{+}$-ion; quantifier)
  & VLSPQGGGPWDSVAR ($y12^{++}$-ion; qualifier)
- 3 transitions per peptide measured
- 10 min gradient (3→33% 0.1% FA in ACN)

Klont et al., Anal. Chim. Acta, **2018**, 1043, 45-51

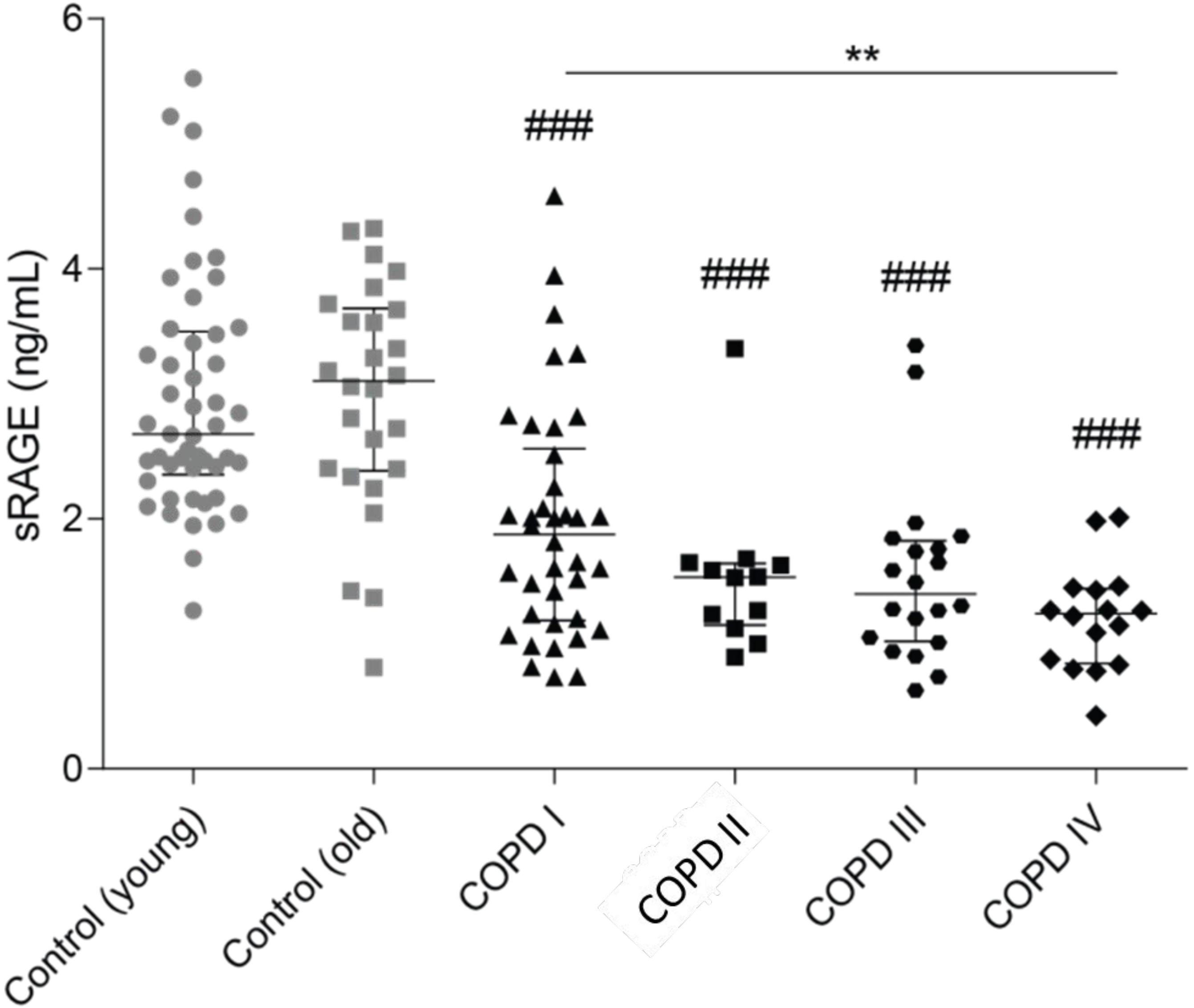
LC-MS of sRAGE in Serum after SCX-SPE

Immunoaffinity **SCX-SPE** **SCX-SPE + TCA**



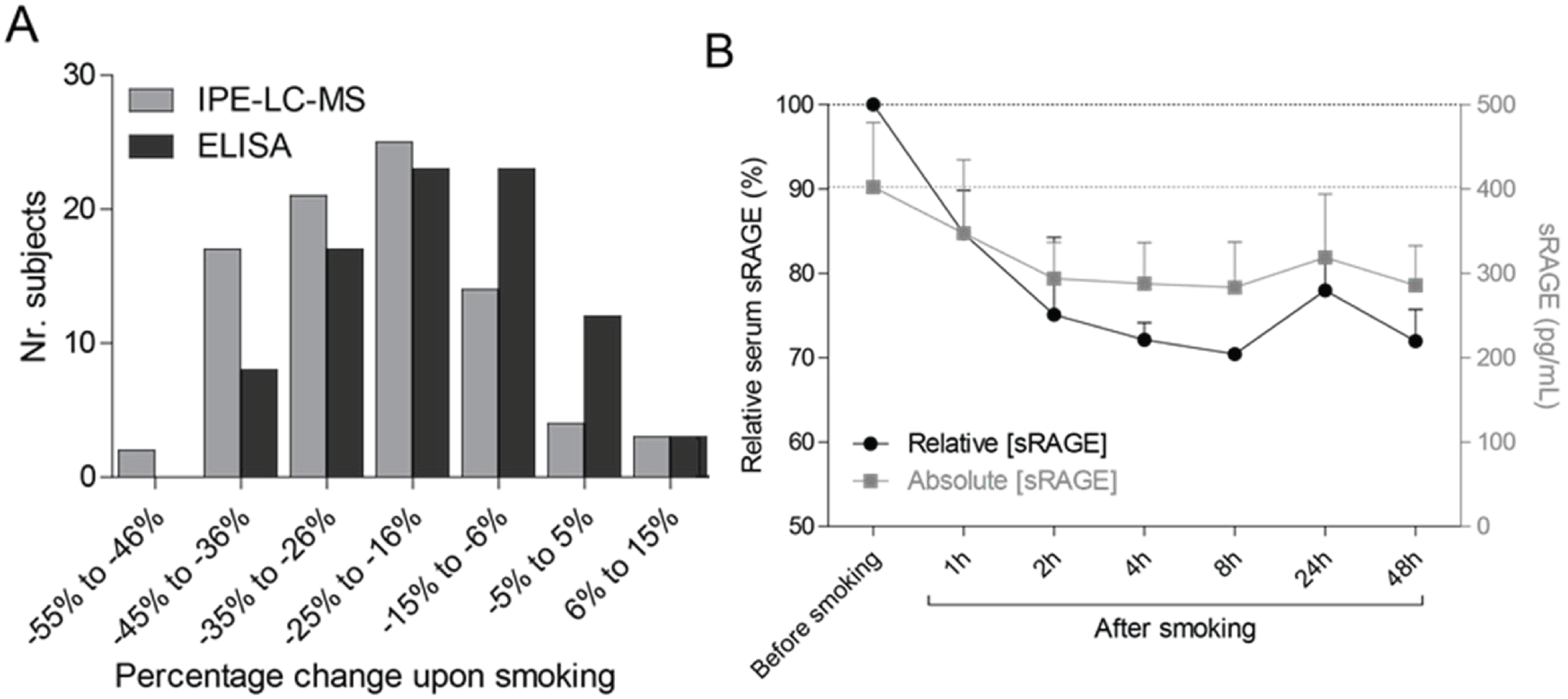
Klont et al., Anal. Chim. Acta, **2018**, 1043, 45-51

Serum sRAGE: Disease Severity



Pouwels et al., *Am. J. Respir. Dis. Crit. Care Med.*, **2018**, 198, 1456-1458

Serum sRAGE and Acute Smoke Exposure



Pouwels et al., *Am. J. Respir. Dis. Crit. Care Med.*, **2018**, 198, 1456-1458

Summary & Conclusions

- Proteins must be treated as family of molecules. Different ‘proteoforms’ may respond differently in LC-MS and in Ligand Binding Assays.
- Endogenous interaction partners (e.g. antibodies, binding proteins) may interfere with affinity-based enrichment strategies prior to LC-MS or Ligand Binding Assays.
- *In vivo* biotransformation of therapeutic proteins will likely affect their pharmacology. This may be different from one patient to another and should be monitored.
- Low levels of biomarkers may be analyzed by LC-MS using different affinity agents but also after more generic sample preparation.

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http://biomarkerdevelopmentcenter.nl/

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Thank you for your attention!